Costimulation Blockade in Renal Allograft Rejection

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Auch aus Steinen, die Dir in den Weg gelegt werden, kannst Du etwas Schönes bauen.

Erich Kästner

Contents

Abbreviations

APC	antigen presenting cell
CML	cell-mediated lympholysis
Cr	^{51}Chromium
CTLA-4	cytotoxic T lymphocyte antigen 4
d	day
DAG	diacylglycerol
DC	dendritic cell
DNAM-1	DNAX accessory molecule-1
DST	donor-specific transfusion
EAE	experimental autoimmune encephalomyelitis
FACS	fluorescence-activated cell sorting
HLA	human leukocyte antigen
ICAM-1	intercellular adhesion molecule-1
ICOS	inducible costimulator
IFN	interferon
IL	interleukin
IP_3	inositol triphosphate
LFA-1	lymphocyte function associated antigen-1
MACS	magnetic cell separation
MAPK	mitogen activated protein kinase
MFI	mean fluorescence intensity
MHC	major histocompatibility complex
MS	multiple sclerosis
mTOR	mammalian target of rapamycin
necl	nectin like
NFκB	nuclear factor κB
NFAT	nuclear factor of activated T cells
PD-1	programmed cell death-1
PBS	phosphate buffered saline
PIP_2	phosphatidylinositol bisphosphate
PKC	protein kinase C
PLC-γ	phospholipase C-γ
PMA	phorbol$_1$2-myristate$_1$3-acetate
PTLD	post-transplant lymphoproliferative disorder
qPCR	quantitative PCR
rTEC	renal tubular epithelial cell
SLE	systemic lupus erythematosus
STAT	signal transducer and activator of transcription
TCR	T cell receptor
Th	T helper cell
TLR	toll-like receptor
TNF	tumor necrosis factor
TNF-R	tumor necrosis factor-receptor
TRAF	TNF-R associated factor

Summary

Kidney transplantation is the best therapy option for patients with end stage renal disease. But patients receiving a graft from a genetically non-identical donor are at a constant risk of rejection. The actual treatment for an organ recipient therefore aims to induce a systemic immunosuppression, which is very potent in inhibition of acute rejections. The long-term outcome of renal transplants, however, is still hampered by the toxicities of these regimens and their disability to prevent chronic allograft rejection. Furthermore, the systemic immunosuppression leads to a higher risk for infections and/or cancer. Therefore, new strategies for more specific and less toxic treatments for recipients of renal grafts are needed. A major goal of research is the induction of donor-specific tolerance. A promising tool to achieve both is costimulation blockade.

Costimulation in addition to recognition of the cognate antigen is needed to fully activate a T cell. A variety of costimulatory pathways are described and their blockade has been shown to be beneficial in models for solid organ transplantation. Costimulation is also important during the interaction of a T cell with its target. Renal tubular epithelial cells (rTECs), which are the major target of cytotoxic T lymphocytes during kidney allograft rejection, do also express costimulatory molecules under inflammatory conditions. One of the most important costimulatory pathways in both processes is the CD40-CD154 pathway. A more recently described costimulatory molecule expressed on T cells is DNAM-1.

In **section 1** we evaluate the role of CD40 expressed on donor cells for the direct alloresponse during the priming and the effector phase *in vitro*. We show that donor CD40 is important for induction of allospecific Th1, Th17 and cytotoxic T cells. In an *in vivo* model for renal allograft rejection we show that renal allografts from CD40 deficient donors are partially protected from the effects of acute rejection. Studies blocking the CD40-CD154 pathway so far mostly used blocking antibodies against CD154. The translation of such an agent to the clinics has resulted in thromboembolic events in patients and is thus not pursued anymore. In mice the blockade of CD40 itself however is not possible with the antibodies available today. In **section 2** we therefore present the development of an F(ab)-fragment against murine CD40 with exclusively antagonistic properties. F(ab)86 is able to prevent CD40 triggered B cell activation and proliferation and reduces allospecific T cell proliferation *in vitro*. DNAM-1 is a costimulatory molecule expressed on T and NK cells. Its two ligands CD155 and CD112 are expressed on several cell types, also in the kidney. In **section 3** we show that DNAM-1 ligands are expressed on rTECs. We detected a role for DNAM-1 during allospecific T cell priming but not during cytotoxic activity against rTECs. Furthermore, we could exclude that ligation of CD155 and CD112 enhances T cell activity during allospecific priming or cytotoxicity against rTECs. The *in vitro* results are further strengthened by the finding that renal allografts deficient in CD155 and CD112 are not protected from rejection. Summing up, this study gives a deeper insight into the role and relevance of the CD40 and DNAM-1 pathways during renal allograft rejection and suggests a new tool to block murine CD40.

Zusammenfassung

Transplantation ist die beste Therapie für Patienten mit Nierenversagen. Aber Patienten die ein Transplantat von einem genetisch unterschiedlichen Spender erhalten tragen ein dauerhaftes Risiko, ihr Transplantat abzustoßen. Die momentane Strategie, um das zu verhindern, ist eine systemische Immunsuppression, welche akute Abstoßungen erfolgreich zu verhindern vermag. Die verwendeten Medikamente besitzen jedoch toxische Nebenwirkungen und sind nicht in der Lage chronische Abstoßungen zu verhindern. Weiterhin tragen diese Patienten aufgrund der Immunsuppression ein erhöhtes Risiko für Infektionen und/oder Krebserkrankungen. Es ist also notwendig, gezieltere und weniger toxische Therapieoptionen zu finden. Eine weitere Option ist die Induktion von immunologischer Toleranz spezifisch für das transplantierte Organ. Eine mögliche Strategie, um beide Ziele zu erreichen, ist die Costimulations-Blockade.
T Zellen benötigen für eine vollständige Aktivierung nicht nur ein Signal durch den T-Zell-Rezeptor, sondern zusätzlich costimulatorische Signale. Mehrere costimulatorische Signalwege sind inzwischen beschrieben. Sie zu blockieren hat sich in mehreren Modellen für Organtransplantation als förderlich erwiesen. Doch auch für die Interaktion zwischen T Zelle und Zielzelle spielt Costimulation eine Rolle. Renale Tubulus-Epithelzellen (rTECs) sind Zielzellen für allospezifische zytotoxische T Zellen während der Abstoßung eines Nierentransplantats. Sie exprimieren costimulatorische Moleküle unter inflammatorischen Bedingungen. Ein wichtiger costimulatorischer Signalweg für beide erwähnten Vorgänge ist der CD40-CD154-Signalweg. Weiterhin wurde DNAM-1 als costimulatorisches Molekül auf T Zellen identifiziert.
In **Abschnitt 1** untersuchen wir die Rolle von CD40 auf Spender-Zellen während der T Zell Aktivierungs- und Effektorpahse *in vitro*. Wir zeigen, dass Donor-CD40 eine Rolle für die Induktion von Th1, Th17 und zytotoxischen T Zellen spielt. In einem *in vivo*-Modell für Nierentransplantation fanden wir weiterhin, dass Transplantate von CD40-defizienten Spendern trotz akuter Abstoßung eine bessere Funktion aufweisen. Die meisten Studien, in denen der CD40-Signalweg bisher blockiert wurde, basierten auf der Nutzung eines Antikörpers gegen CD154. Als ein solcher Antikörper zum ersten Mal in Patienten verwendet wurde, traten jedoch thromboembolische Komplikationen auf, die eine solche Therapie unmöglich machen. In Mausmodellen ist es bisher nicht möglich gewesen CD40 selbst zu blockieren, da alle verfügbaren Antikörper agonistisch wirken. In **Abschnitt 2** zeigen wir daher die Entwicklung eines F(ab)-Fragments gegen Maus-CD40, das ausschließlich antagonistische Eigenschaften besitzt. F(ab)86 war *in vitro* in der Lage CD40-mediierte B Zell Aktivierung und allospezifische T Zell-Proliferation zu supprimieren. DNAM-1 ist ein costimulatorisches Molekül, das auf T und NK Zellen exprimiert wird. Seine zwei Liganden CD155 und CD112 werden auf verschiedenen Zelltypen exprimiert, unter anderem in der Niere. In **Abschnitt 3** zeigen wir, dass CD155 und CD112 auf rTECs exprimiert werden. Wir konnten weiterhin feststellen, dass DNAM-1 eine Rolle während der allospezifischen T Zell Aktivierung spielt. Für die Zytotoxizität gegen rTECs hingegen wird DNAM-1 nicht benötigt. Letzt-

lich konnten wir ausschließen, dass die beiden Liganden CD155 und CD112 einen Einfluss auf allospezifische Aktivierung oder Zytotoxizität gerichtet gegen rTECs haben. Diese *in vitro* Ergebnisse konnten wir in einem *in vivo*-Modell erhärten. Nierentransplantate von CD155- oder CD112-defizienten Spendern sind nicht vor der Abstoßung geschützt.
Zusammenfassend können wir mit dieser Arbeit die Rolle von CD40 und DNAM-1 während der Abstoßung von Nierentransplantaten genauer erklären und eine Strategie präsentieren, um murines CD40 zu blockieren.

Chapter 1

Introduction

Renal transplantation

Renal transplantation is the best therapy option for patients with end-stage renal disease (ESRD) (Wolfe *et al.* 1999). Major causes for ESRD are diabetes, hypertension, and glomerulonephritis (USRDS 2010 Annual Data Report). Diabetes and hypertension are both diseases with a high prevalence, especially in developed countries. The estimated number of diabetes patients all over the world in the year 2000 was 171 million, with a rising tendency – 366 million cases are expected by the year 2030 (Wild *et al.* 2004). Patients with diabetic nephropathy develop glomerular hypertrophy and microalbuminuria. They present with a higher glomerular filtration rate in the beginning, due to higher intraglomerular capillary pressure. However, eventually the GFR decreases because of nodular glomerusclerosis and interstitial injury, which are most likely exacerbated by hypertension and poor glycemic control (Maric and Hall 2011).
Also hypertension is a widespread condition with about 972 million patients all over the world (Hajjar *et al.* 2006) and a prevalence of 26% (Kearney *et al.* 2005). The mechanism how high blood pressure leads to renal damage and failure is not completely understood. Most probably the damage of the renal vasculature leads to further damage in the glomeruli, which then lose their ability to prevent hyperfiltration injury. Furthermore, the glomeruli show signs of hypoxia (reviewed in Udani *et al.* 2011).
Glomerulonephritis is a wide definition of several inflammatory diseases affecting the glomeruli including minimal change disease, focal segmental glomerulosclerosis, or membranous glomerulonephritis. IgA nephropathy is the most common primary glomerulonephritis. This condition is characterized by the deposition of immune complexes in the kidney. These immune complexes consist of antibodies of the IgA1 subclass, which show a lack of galactose in the O-linked glycosylations of the hinge region of the heavy chain. Pathologic complexes of circulating IgG antibodies against these abnormally glycosylated IgA molecules are very big (800 kDa) and deposited in the mesangium of the glomeruli. Mesangial cells in turn become activated and react by proliferation and extracellular-matrix deposition. (Reviewed in Suzuki *et al.* 2011)
Other diseases leading to loss of renal function are for example polycystic kidney disease and immunologic diseases like lupus nephritis. Polystic kidney disease is an inherited condition with a prevalence of 1 case in 1'000 population (Torres *et al.* 2007). It is characterized by an extensive development of cysts lined by epithelium, which destroys the renal architecture. The genes affected in autosomal dominant polycystic kidney disease are encoding for PC-1 and PC-2. The loss of function of one or two of these genes leads to a lack of control in cell proliferation and apoptosis and a loss of polarity and differentiation of affected cells. Up to today there are no treatment options for polycystic kidney disease. (Park *et al.* 2011)
Systemic lupus erythematosus (SLE) is an autoimmune disease, which can affect several organs. Involvement of the kidney leads to significant morbidity. The renal damage in lupus nephritis is mainly caused by the deposition of immune complexes in the glomeruli, complement activation, and interstitial infiltrates

of activated lymphocytes (Apostolidis *et al.* 2011). The prevalence of SLE is 20 to 150 cases per 100'000 population, depending on the region (Tsokos 2011). The first treatment option for patients with a loss of renal function and no matching organ donor is dialysis. Generally speaking, in hemodialyisis the patient's blood is filtered externally against a dialysis solution across a semipermeable membrane, which allows molecules and water to diffuse, but restricts lager molecules like vitamin B12 and albumin (Hamilton 1999). In peritoneal dialysis the patient's peritoneum is used as semipermeable membrane and the blood in the capillaries is filtered against a dialysis solution, which is infused in the peritoneal cavity (Khanna and Nolph 1999). Both types of dialysis attempt to replace renal function by removing metabolic waste products like urea and creatinine and by diffusion of extrinsic bicarbonate to the patient's blood in order to correct metabolic acidosis. However, dialysis is not able to replace the endocrine functions of the kidney, like production of erythropoietin and calcitriol (Hamilton 1999). Furthermore, the risk of developing cardiovascular diseases is much higher in patients on dialysis compared to the normal population. The prevalence of cardiac failure is approximately 40 % (Foley *et al.* 1998). Salt and water overload and anemia in dialysis patients lead to a volume overload of the left ventricle and in combination with hypertension and arteriosclerosis enhance the risk of cardiovascular events (Parfrey and Foley 1999). Thus, even though dialysis efficiency and safety have been improved over the years, transplantation is still the best therapy for ESRD – even though early after transplantation the risk of death is higher compared to patients that stay on dialysis. However, at 18 months after transplantation the relative risk of death is as low as 0.32 compared to a dialysis population and the expected lifespan of the patients increases by 10 years (Wolfe *et al.* 1999).

Despite these impressive outcome results, patients carrying a renal allograft are at a constant risk of organ rejection. In order to prevent this, they are usually treated with a cocktail of immunosuppressive drugs consisting of corticosteroids, calcineurin inhibitors (cyclosporine A or tacrolimus) and purine biosynthesis inhibitors (mycophenolate mophetil). With support of these drugs and induction therapies with T cell-depleting (anti-thymocyte globulin) or -inhibiting (basiliximab) antibodies, the incidence of acute rejections and early graft loss was markedly reduced during the last decade. However, chronic rejection and toxicities of the treatment are still hampering the long term survival of renal allografts (Fig. 1.1) (Hariharan *et al.* 2000). Cyclosporine A for example has been shown to cause hyperlipidemia, hypertension and new onset diabetes (Curtis 2002; Guitard *et al.* 2011; Kobashigawa and Kasiske 1997). Furthermore, cyclosporine A has a nephrotoxic effect leading to allograft fibrosis and reduced renal function (Tedesco and Haragsim 2012). The alternative calcineurin inhibitor tacrolimus has been shown to be more effective in preventing acute rejection and promoting allograft survival during the first year compared to cyclosporine A (Webster *et al.* 2005). However, it shares most of the side effects with its predecessor (Margreiter 2002; Sperschneider 2001). Mycophenolate mofetil on the other hand is associated with gastrointestinal

side effects, which limit tolerability and reduce efficacy due to necessary dose adjustments (Knoll *et al.* 2003). Finally, systemic immunosuppression is associated with higher rates of infections and cancer possibly leading to death with a functioning graft (Busnach *et al.* 2006; Dunn 1990).

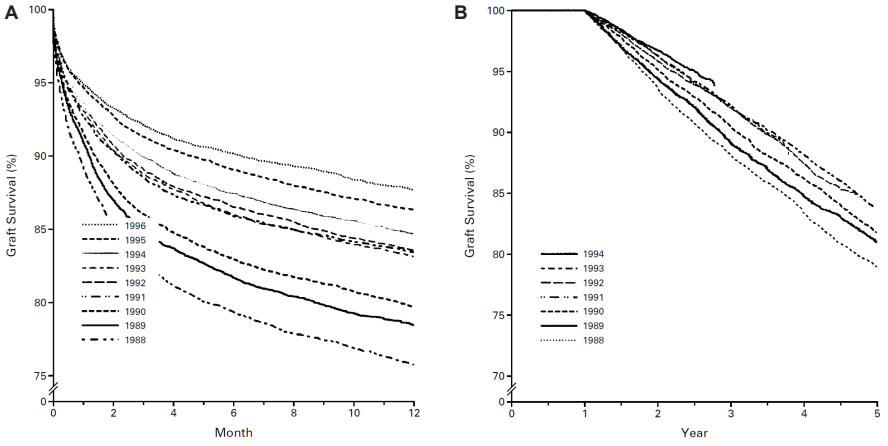

Figure 1.1: Development of renal allograft survival 1988 - 1994. Survival of renal allografts has improved significantly during this time period (A). Long term allograft survival, however, has not changed markedly (B). Figure adapted from Hariharan *et al.*, 2000

Antigens and antigen presentation

Major histocompatibility antigens

Major histocompatibility complex (MHC) molecules are divided in two major subclasses - class I and class II. Both groups show a closely related overall structure with the major feature being the cleft in which peptides derived from self- and non-self molecules are presented. MHC molecules are essential for the presentation of antigenic peptides to T cells. MHC class I molecules consist of a membrane spanning α-chain and a β2-microglobulin. On this complex, which is expressed ubiquitously on all nucleated cells of the body, peptides derived from proteasomal degradation are presented to CD8 T cells. MHC class II molecules are assembled of a non-covalent complex of two transmembrane proteins called α- and β-chain. This complex is responsible for the presentation of peptides derived from extracellular proteins to CD4 T cells. MHC class II is expressed on professional antigen presenting cells (APCs) such as dendritic cells (DCs), macrophages, and B cells.

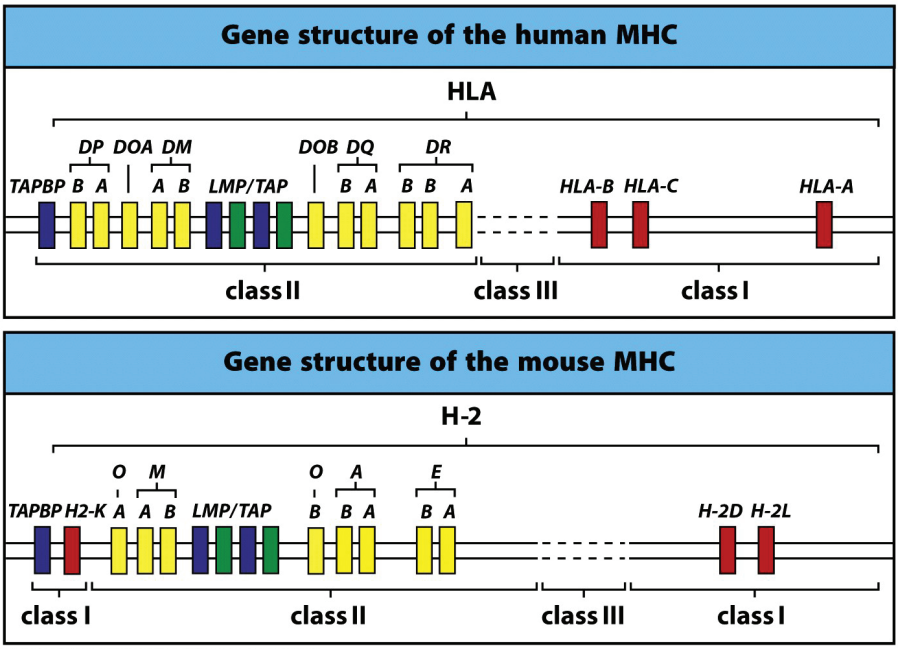

Figure 1.2: Gene structure of the human and murine MHC locus.
Both contain genes for MHC I and MHC II molecules plus additional genes encoding for accessory molecules. Figure adapted from Janeway's Immunobiology, 7th edition, 2008

The polygenic MHC is located on chromosome 6 in humans and on chromosome 17 in the mouse. It contains several gene loci for MHC class I and class II genes. These genes are called human leukocyte antigens (HLA) in humans and H-2 genes in mice (Fig. 1.2). There are three different MHC class I gene loci in humans: HLA-A, -B, and -C. The gene loci for MHC class II are called HLA-DR, -DP, and -DQ. In mice there are three different genes encoding for MHC class I (H-2K, -D, and -L) and two genes for MHC class II (H-2A, -E). The gene products of MHC I and II are highly polymorphic. There are more than 400 alleles of some human MHC class I and class II gene loci and most human individuals will be heterozygous for them. Expression of the MHC loci is codominant. Thus, polygeny and polymorphism of the MHC lead to a high variability of potential peptides presented. This variety is a big evolutionary advantage concerning the diversity of pathogens a population might encounter. In transplantation, however, it is one of the major obstacles. This becomes most evident, when comparing survival of renal allografts from HLA-identical siblings to those from HLA-non-identical ones. The latter survive significantly shorter (Colvin and Nickeleit 2006). Furthermore, the outcome of human renal allografts is dependent on the amount of MHC mismatches in the genes HLA-A, -B and -DR (Opelz 1997). In mice mutations of one to three amino acids in single MHC molecules are sufficient to induce allograft rejection (Cornell *et al.* 2008).

ABO-antigens

The ABO system discriminates between three blood groups: A, B and O. The ABO antigens are carbohydrate epitopes present on different core saccharide chains bound to lipids or proteins. Blood group A is characterized by the terminal trisaccharide GalNacα1-3[Fucα1-2]Galβ, blood group B carries Gal$\alpha\alpha$1-3[Fucα1-2]Galβ and blood group C Fucα1-2Galβ. ABO antigens are ubiquitously expressed on almost all cells of the body (Szulman 1960). According to a rule postulated by Karl Landsteiner, humans have antibodies to those ABO antigens not present in their own body (Landsteiner 1945). In the 1950s and 60s the first attempts to transplant kidneys across the ABO-antigen barrier (e.g. from an A-donor to an O-recipient) led to hyperacute rejection episodes within minutes after transplantation (Starzl *et al.* 1964). Nowadays more and more kidney transplantations across the ABO-barrier are performed. However, in this case patients have to undergo a pretreatment attempting to reduce the amount of circulating antibodies against the antigen to be transplanted. This is achieved by plasmapheresis, adsorption on protein A or immunoadsorption on columns with specific carbohydrate antigen epitopes covalently linked to a solid phase. (Reviewed in Shin and Kim 2011) A phenomenon called accommodation occurs in many of these patients transplanted in the presence of low levels of anti-graft antibodies. In this case the A or B antigens are still expressed on the allograft and *de novo* produced antibodies bind to them, as shown by complement fixation in the graft. However, for an unknown reason this does not lead to allograft injury. Thus, accommodation is defined as the survival of an allograft in the presence of specific antibodies and complement, which would otherwise lead to graft rejection. (Reviewed in Dehoux and Gianello 2009)

Minor histocompatibility antigens

Minor histocompatibility antigens are immunogenic peptides derived from polymorphic proteins presented in the context of host HLA (Dierselhuis and Goulmy 2009). The role of minor histocompatibility antigen differences has been well described in the setting of HLA-identical stem cell transplantation (Goulmy *et al.* 1996). Differences in minor histocompatibility antigens can also induce renal allograft rejection in an HLA and blood group matched situation - for example in HLA-identical sibling donor-recipient-pairs (Dierselhuis and Goulmy 2009). The most intensively studied group of such antigens is the one encoded on the Y-chromosome, which is only present in male individuals. HY antigens are in most cases ubiquitously expressed on renal proximal tubular cells (de Bueger *et al.* 1992). In a big multicenter study it was shown, that renal allograft survival was significantly reduced in women receiving a male organ (Gratwohl *et al.* 2008). According to this study the HY-mismatch can lead to acute as well as chronic rejection. It has furthermore been shown, that *de novo* antibodies directed against HY antigens correlate with incidence of acute rejection and plasma cell infiltration (Tan *et al.* 2008). The influence of other minor histocompatibility antigens on renal allograft survival is much less studied.

Ways of allopresentation

Alloreactive T cells can recognize their target in either the indirect, semi-direct, or direct manner. In indirect allo-recognition, T cells recognize peptides derived from donor MHC molecules presented on recipient APCs (Shoskes and Wood 1994). The indirect allo-reaction is comparable in strength to any immune reaction directed against a non-self peptide (Afzali *et al.* 2008; Womer *et al.* 2001). It takes place, when recipients APCs take up donor antigen from the transplanted organ and present it to the cognate T cell in the draining lymph node. Furthermore, a *de novo* B cell response is dependent on indirectly alloreactive CD4 helper T cells, which recognize peptides derived from antigens internalized by the B cells and presented in the context of MHC II (Taylor *et al.* 2007).
Host APCs can also semi-directly present intact donor MHC molecules, which they have taken up via cell-cell contact or the incorporation of exosomes (Herrera *et al.* 2004). Directly alloreactive T cells on the other hand recognize intact foreign MHC molecules presented by donor APCs, which migrate from the transplanted organ to the draining lymph node (so called passenger-leukocytes). Direct allo-recognition leads to a much stronger response than the indirect pathway (Liu *et al.* 1993); also because of a high frequency of directly allospecific T cells, which is estimated to be around 10% of all T cell clones (Lindahl and Wilson 1977; Sherman and Chattopadhyay 1993). The reason for this high precursor frequency is still not clear. Two theories arose to explain this phenomenon. The "high density determinant"-theory argues, that directly alloreactive T cells recognize polymorphic epitopes on the donor-MHC molecules themselves independent of the peptide presented. The "determinant frequency"-theory assumes, that the T cell receptors (TCRs) of directly alloreactive T cells recognize a variety of foreign peptides presented on donor-MHC molecules. In this case the T cell response arises not only against peptides different in sequence but also to those different in conformation, when bound to allo-MHC molecules. (reviewed in Archbold *et al.* 2006) Both mechanisms do not exclude each other and it is likely, that both take place depending on the site and magnitude of the structural differences in MHC molecules between donor and recipient (Afzali *et al.* 2008).

Renal allograft rejection

The incidence of acute cellular rejection in unsensitized patients is nowadays 5 - 10 % in the first year (Cornell *et al.* 2008). Pathologically acute rejection presents with infiltrates of mononuclear cells in the interstitium and inflammation of the tubules and in some cases also the arteries (Colvin and Nickeleit 2006). The interstitial infiltrates consist mostly of CD4 and CD8 T cells (Colvin and Nickeleit 2006). Another characteristic feature of acute cellular rejection is the development of tubulitis, an invasion of the tubular epithelium by infiltrating T cells and macrophages (Colvin and Nickeleit 2006). A prominent cytokine found in acutely rejected allografts is IFN-γ, which is secreted by T helper 1 and cytotoxic CD8 T cells (Hoffmann *et al.* 2005). Also interleukin

(IL)-17 has been found in early renal allograft rejection in human urine samples and an experimental model for acute rejection in the rat (Loong *et al.* 2002). Both cytokines are known to activate renal tubular epithelial cells and thereby induce changes in surface and chemokine expression (Starke *et al.* 2007; Woltman *et al.* 2000). In experimental studies it has been shown, that the T cells responsible for acute rejections are mostly directly alloreactive (Benichou *et al.* 1999).
Also B cell-dependent mechanisms can lead to allograft rejection. Hyperacute rejection is mediated by preformed antibodies against antigens expressed on the allograft. It is a strong immune response leading to graft loss already minutes to hours after transplantation. This complication is mostly avoided by HLA- and blood group-matching and pretransplant cytotoxic crossmatch testing (Bohmig *et al.* 2002). However, approximately 25% of acute rejections are at least in part due to a humoral component (Cornell *et al.* 2008). Acute humoral rejection can occur days to weeks or years after transplantation. Pathologically it is recognizable by detection of deposits of C4d in the peritubular capillaries. C4d is an inactive fragment of C4b, a part of the classic complement pathway. It has no known function itself but remains bound in the tissue for several days after complement activation by specifically bound antibodies. (Reviewed in (Cornell *et al.* 2008)) Even though $88 - 95\%$ of patients presenting with C4d deposits have anti donor-HLA antibodies (Colvin 2007), these are not necessarily detectable in the circulation – probably due to the absorption of the alloantibodies by the graft (Martin *et al.* 2005).
Chronic allograft rejection can be cellular or humoral or both (Cornell *et al.* 2008). Pathologic features of chronic renal allograft rejection are chronic changes such as transplant glomerulopathy, peritubular capillaropathy, transplant arteriopathy, interstitial fibrosis, and tubular atrophy (Colvin and Nickeleit 2006). Experimental studies have suggested that T cells mediating chronic rejection are mostly activated by the indirect pathway (Benichou *et al.* 1999).

Costimulation and Coinhibition

Three signals are necessary for T cell activation

Antigen presenting cells deliver more than one signal to T cells in order to activate them. At least three signals are needed to achieve a fully functional T cell. Signal one is delivered by the interaction of the TCR with its cognate antigen presented in the context of self-MHC. In the case of direct allo-recognition this signal can also be delivered by a foreign MHC molecule, as discussed above. The additional binding of CD4 to MHC class II and CD8 to MHC class I, respectively, is necessary, but not sufficient to induce T cell activation and proliferation. Signal 1 leads to the activation of phospholipase C-γ (PLC-γ). PLC-γ in turn cleaves phosphatidylinositol bisphosphate (PIP_2) into diacylglycerol (DAG) and inositol triphosphate (IP_3). DAG leads to activation of kinases like protein kinase C (PKC), which lead to activation of the transcription factors nuclear factor κB (NFκB) (Cheng *et al.* 2011) and via the Ras-mitogen activated protein kinase (MAPK) cascade AP-1. IP_3 increases the intracellular Ca^{2+}

concentration. This activates a serine phosphatase called calcineurin, which in turn activates a third transcription factor named nuclear factor of activated T cells (NFAT), which can only enter the nucleus in a dephosphorylated state. These three transcription factors bind to the promoter region of the IL-2 gene and are essential for its transcription. IL-2 is an essential autocrine cytokine for the survival of activated T cells. However, a signal 2 (a so called co-stimulatory signal) is needed to increase IL-2 signaling. This signal is for example delivered by the costimulatory receptor CD28 expressed on the T cell. CD28 is the best characterized and probably most important costimulatory receptor and binds to B7-1 (CD80) or B7-2 (CD86) presented by the APC. The triggering of CD28 leads to an increase in the nuclear translocation of AP-1 and NFκB, which enhances the initiation of transcription of IL-2. Furthermore, the stability of IL-2 mRNA is increased. Both effects lead to a much higher IL-2 protein production (signal 3). Naive T cells express a receptor for IL-2 consisting of a β- and a γ-chain. This receptor binds IL-2 only with a moderate affinity. The delivery of a costimulatory signal also induces the expression of the α-chain, which gives the IL-2 receptor a high affinity for its ligand.

Costimulatory pathways

In addition to CD28-B7 there is a variety of costimulatory receptor-ligand pairs described (summarized in Fig. 1.3). A further costimulatory receptor is inducible costimulator (ICOS, CD287) which binds to ICOS-ligand (ICOS-L). ICOS-L is expressed on activated DCs, monocytes and B cells. ICOS does not induce IL-2 production, but regulates the expression of other cytokines produced by CD4 subsets like IFN-γ, TNF-α, IL-4, IL-5, and IL-10 (Hutloff *et al.* 1999). Mice deficient in ICOS are not capable to control viral or worm infections owing to impaired Th1 and Th2 responses, respectively (Kopf *et al.* 2000). ICOS-ICOS-L interaction also plays a role in the development of follicular T helper cells and their interaction with B cells. Mice lacking ICOS show impaired germinal center formation and antibody class switching (reviewed in Simpson *et al.* 2010).
CD27 belongs to the TNF-receptor family and is constitutively expressed on naive T cells. It binds to CD70 on DCs and gives a strong co-stimulatory signal early in the activation process. CD27 signaling supports T cell survival rather than the entry into the cell cycle (reviewed in Denoeud and Moser 2011).
4-1BB (CD137) and 4-1BBL (CD137 ligand) is another pair of the TNF-receptor and -ligand family. 4-1BBL is expressed on activated DCs, macrophages and B cells. The interaction of these two molecules is bidirectional and delivers activating signals to both the T cell and the APC (reviewed in Shao and Schwarz 2011).
OX40 and OX40L also belong to the TNF-receptor/TNF-ligand family. OX40 is expressed on activated T cells (Gramaglia *et al.* 1998). Its ligand is expressed on activated DCs, B cells and vascular epithelial cells (Stuber and Strober 1996). Their interaction plays a critical role in Th1/Th2 differentiation as well as memory T cell generation (Lane 2000; Rogers *et al.* 2001). Furthermore,

OX40 is constitutively expressed on regulatory T cells (Treg). In these cells OX40 ligation leads to decreased FoxP3 expression and loss of suppressor function (Vu *et al.* 2007).
The receptor-ligand pair CD40-CD154 (CD40L) will be described in more detail in the following section of this chapter.
If a T cell receives signal 1 in the absence of signal 2, for example via CD28, it either undergoes apoptosis or reaches a state of anergy. Anergy is defined as a state in which the T cell is not able to be activated anymore, even if it receives signal one in combination with co-stimulation at a later time point. This process is important to maintain self-tolerance. If a T cell was activated without a costimulatory signal, auto-reactive T cells would be easily activated in the periphery by different kinds of cells.

Coinhibitory pathways

Another way of controlling a T cell response is the ligation of coinhibitory receptors, such as cytotoxic T lymphocyte antigen 4 (CTLA-4, CD152) or programmed cell death-1 (PD-1). CTLA-4, an additional receptor for the B7 molecules, is induced on activated T cells. It binds B7-1 and B7-2 with a twenty times higher avidity than CD28. Thus, CTLA-4 reduces binding of CD28 to its ligands and deliverers an inhibitory signal to the T cell itself. This leads to a reduced production of IL-2 and subsequently to less T cell proliferation (Walunas *et al.* 1996). Mice lacking CTLA-4 develop a multiorgan disease characterized by a strong overgrowth of lymphocytes (Tivol *et al.* 1995; Waterhouse *et al.* 1995).
PD-1 is induced transiently on activated T cells. One of its two ligands PD-L1 (B7-H1) is expressed on a wide variety of cells including T cells, B cells, DCs, and macrophages but also on nonhematopoetic cells like vascular endothelial cells or renal tubular epithelial cells (Fife and Pauken 2011). The other ligand PD-L2 (B7-DC) is induced on APCs during inflammation. PD-1/PD-L1 interactions have been shown to play a role in regulating autoreactive T cells specific for tissue-restricted self-antigens (Fife and Pauken 2011). PD-1/PD-L2 interactions in contrast are critically involved in regulating immune responses to environmental antigens (Fife and Pauken 2011). Mice lacking PD-1 develop autoimmune diseases, because they lack the ability to regulate T cell activation (Nishimura *et al.* 1998; Nishimura *et al.* 1999; Nishimura *et al.* 2001). Recently, it has been shown that the B7-1 molecule expressed on the surface of T cells serves also as receptor for PD-L1 and thus also transmits negative signals to T cells (Butte *et al.* 2007). This suggests that PD-1 might not be the unique mediator of negative signals delivered by PD-L1.

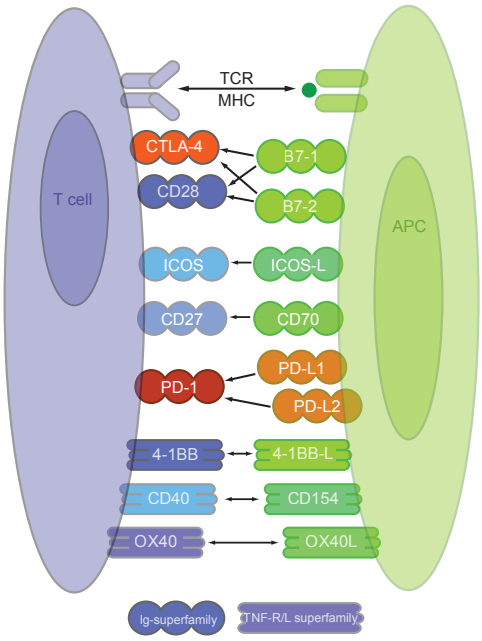

Figure 1.3: Costimulatory and -inhibitory molecules. T cells need additional signals to the one mediated by the T cell receptor in order to be fully activated. A secondary signal can be given via costimulatory receptors. Here a variety of these molecules expressed on the T cell and their respective ligands expressed on the APC are depicted (receptors in blue, ligands in green). Furthermore, there are receptors that negatively regulate the T cell response (red). The depicted receptors either belong to the Immunoglobulin (Ig)- or the TNF-receptor-superfamily. The ligands on the other hand either belong to the Ig- or the TNF-receptor-ligand-superfamily.

T helper cell differentiation

CD4 positive T helper (Th) cells can differentiate into different subsets. In order to do so they need a third signal from the APC. Signal 3 is delivered by cytokines that drive the T cell to one or the other direction. T helper subsets are defined by a characteristic transcription factor and specific set of cytokines secreted (summarized in Fig. 1.4). The first two T helper cell subsets described are Th1 and Th2. Th1 cells are characterized by the transcription factor T-bet. They produce IL-2 and IFN-γ. For their induction they require IL-12 and IFN-γ as signal 3. This Th subset plays a major role in the clearance of acute infections. Furthermore, it is involved in acute allograft rejection (D'Elios *et al.* 1997). The specific transcription factor for Th2 cells is GATA-3. These cells require IL-4 as third signal and secrete mainly IL-4 and IL-5. Their importance has been shown in chronic immune reactions like allergy. In allograft rejection this Th subset plays a role rather in chronic rejection (Shirwan 1999). A subset of Th cells discovered in 1995 are the regulatory T cells (Treg) (Sakaguchi *et al.* 1995). Treg are characterized by the expression of FoxP3 and their secretion of inhibitory cytokines like TGF-β and IL-10. Treg are essential for regulating immune responses, which is important to keep self tolerance during strong immune

responses to pathogens. Treg need TGF-β as signal 3. TGF-β, however, can also induce another subset of Th cells. If it is secreted simultaneously with IL-6 this rather drives the development of Th17 cells. This Th subset has recently been described (Langrish *et al.* 2005; Park *et al.* 2005) and is characterized by its transcription factor RORγT. Th17 cells produce proinflammatory cytokines like IL-6 and IL-17. Their role in different immune responses is not completely explored yet. They have been shown to be important during infection with extra- and intracellular bacterial pathogens (Raffatellu *et al.* 2008; Ye *et al.* 2001) but also in models for autoimmune diseases like experimental autoimmune encephalomyelitis (EAE) or rheumatoid arthritis (reviewed in (Hu *et al.* 2010)). The role of Th17 cells in allograft rejection is not yet clear and subject of investigations.
Taken together, signal 1 delivered by the TCR is essential for activation, signal 2 delivered by costimulatory receptors for survival, and signal 3 delivered by the cytokine milieu for the differentiation of a T cell.

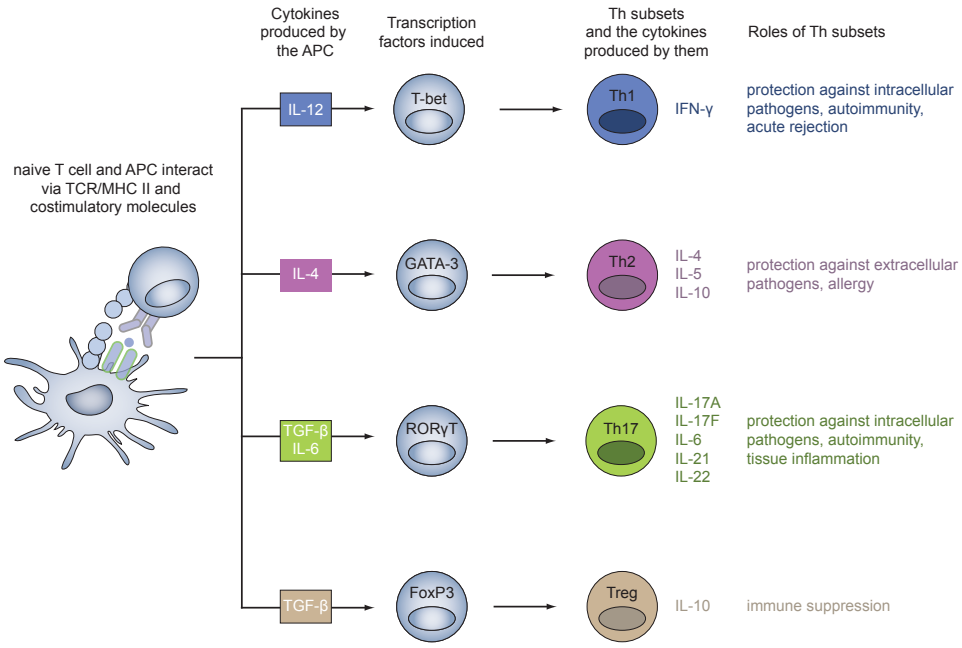

Figure 1.4: Th subsets. Upon interaction with an APC presenting the cognate antigen and providing costimulation, the naive CD4 T cell is driven to one of the indicated Th subtypes through the cytokines provided by the APC. The characteristic transcription factors, cytokine profiles, and roles of the Th subsets are indicated.

The CD40-CD154 pathway

CD40

CD40 is a costimulatory receptor belonging to the TNF-receptor (TNF-R) family. It is a type I transmembrane protein consisting of 277 and 305 amino acids in human and mouse, respectively. The intracellular domain does not show a close relationship to any other characterized molecule. The extracellular domain is homologous to other TNF-receptor family molecules. It contains three cysteine rich domains, of which each has 2 - 3 disulfide bridges (An *et al.* 2011). Human and murine CD40 share 62% amino acid identity. All 22 cystein residues are conserved between the two species indicating, that both CD40 molecules fold into the same protein domains (van Kooten and Banchereau 2000). The gene for CD40 is encoded on chromosome 20 in humans and on chromosome 2 in mice (van Kooten and Banchereau 2000). CD40 is expressed on B cells and DCs, monocytes, platelets, and macrophages. In addition it is also expressed on non-hematopoetic cells such as fibroblasts, epithelial, and endothelial cells (Banchereau *et al.* 1995; Bourgeois *et al.* 2002; van Kooten and Banchereau 1997).
Trimerization of the CD40 receptor leads to recruitment of TNF-R-associated factors (TRAFs) to the cytoplasmic domain of CD40. There are six known TRAFs so far, which can induce different signaling pathways including the canonical and non-canonical NFκB-pathways, the MAPKs, phosphoinositide 3-kinase (PI3K), as well as the PLCγ pathway (Bishop *et al.* 2007). All TRAFs except for TRAF4 have been shown to interact with CD40 either directly or indirectly via other TRAFs (Elgueta *et al.* 2009). Also TRAF-independent signaling via the Janus family kinase (Jak3), which can bind to the cytoplasmic domain of CD40 directly, takes place. This pathway induces the phosphorylation of signal transducer and activator of transcription 5 (STAT5) (Saemann *et al.* 2003; Saemann *et al.* 2002).
The biological consequences of CD40 ligation differ greatly depending on the cell type it is expressed on. Some of these are summarized in table 1.1 (modified from van Kooten and Banchereau 2000). In B cells CD40 induces proliferation, differentiation and antibody production. It has been shown *in vitro* that CD40 activation has a direct effect on cytokine production (IL-6, IL-10, TNF-α, lymphotoxin-α) and surface molecule expression (intercellular adhesion molecule-1 (ICAM-1), CD23, B7-1, B7-2). Furthermore, MHC class I and II are upregulated together with the TAP transporter, which is an important part of the pathway for peptide loading on MHC class I (Khanna *et al.* 1997). Thereby the ability of the B cell to present antigen on MHC and activate T cells is enhanced after CD40 triggering. Furthermore, CD40 ligation is essential for the initiation of isotype switching and somatic hypermutation to enhance antibody affinity in germinal center B cells (Elgueta *et al.* 2009). The cytokine milieu determines the type of antibody produced afterwards. In humans IL-4 and IL-13 induce the switch from IgM to IgE and IgG4. Switch to IgG1 and IgG3 is induced by IL-10 and the determining factor for IgG2 is yet to be found. The switch to IgA is promoted by a combination of IL-10 and TGF-β. These

important functions of the CD40-CD154 pathway are lost in patients with the X-linked immunodeficiency hyper-IgM syndrome. These patients show a genetic alteration of the CD154 gene (Notarangelo *et al.* 1996). They are not able to mount proper T cell-dependent antibody responses or to develop B cell memory, and they have only little circulating class-switched antibodies. This phenotype was reproduced in mice by knocking out the gene for CD40 or CD154 (Kawabe *et al.* 1994; Renshaw *et al.* 1994; Xu *et al.* 1994).
CD40 is also expressed on monocytes and DCs, both professional APCs. CD40 ligation on these cells results in enhanced survival, the secretion of cytokines and enzymes, enhanced monocyte tumoricidal activity, and nitric oxygen synthesis. Furthermore, other costimulatory molecules like ICAM-1, LFA-3, B7-1, and B7-2 are upregulated. Activation of CD40 on DCs also enhances their ability to cross-present antigen (Quezada *et al.* 2004). Therefore CD40 signaling is one of the critical signals for complete DC maturation, driving them to be the most potent APCs (Banchereau and Steinman 1998). The interaction of DCs and CD4 T cells via the CD40-CD154 pathway has been shown to enable the DC to effectively stimulate cytotoxic CD8 T cell responses (Bennett *et al.* 1998; Schoenberger *et al.* 1998). In a mouse model it has furthermore been shown, that the interaction of CD154 on CD8 T cells with CD40 on DCs is necessary to stimulate maximal responses in the absence of CD4 help (Hernandez *et al.* 2007). Along with this, mice lacking CD154 mount reduced T cell responses to both intra-and extracellular pathogens (Campbell *et al.* 1996; Soong *et al.* 1996; Wiley and Harmsen 1995). Some pathogens however deliver other signals (like toll-like receptor (TLR)-signals), which may substitute for the CD40-CD154 crosstalk between DC and CD4 T cell and enable the DC to activate CD8 T cells efficiently (Hamilton *et al.* 2001). This is consistent with the observation, that CD154 deficient mice can mount normal T cell responses to different viruses (Oxenius *et al.* 1996; Whitmire *et al.* 1996).

Table 1.1: Differential effects of CD40 activation on several cell types

Cell type	**Functional consequences**
Pre-B cells	proliferation CD23 expression
Naive mature B cells	proliferation differentiation isotype switch
Germinal center B cells	proliferation differentiation Fas expression selection
Plasma cells	IL-6 production
Monocytes/macrophages	cytokine secretion NO production production of metalloproteinases monocyte procoagulant activity tissue factor expression
Dendritic cells	growth and survival expression of costimulatory molecules enhanced cytokine production
Langerhans cells	see dendritic cells
T cells	proliferation CD25 expression cytokine production
Endothelial cells (HUVEC)	up-regulation of CD54, CD62E, CD106 increased tissue factor/thrombomodulin expression, and proagulant activity T cell costimulation increased production of LIF, IL-6, GM-CSF
Thymic epithelial cells	GM-CSF production
Kidney epithelial cells	cytokine/chemokine secretion: IL-6, LIF, GM-CSF, IL-8, MCP-1, RANTES

CD154

CD154, the ligand of CD40, is a type II transmembrane protein with its C-terminus on the extracellular side of the cell membrane. The extracellular domain contains a TNF-like domain, whose three dimensional organization resembles the one of TNF-α and is responsible for the formation of the active homotrimeric form of the molecule (Karpusas *et al.* 1995). Human and murine CD154 share 78% amino acid identity. The gene for CD154 is located on the X chromosome. CD154 is expressed primarily on activated T cells, but also on activated B cells and platelets. Under inflammatory conditions CD154 expression can also be induced on monocytic cells, natural killer (NK) cells, mast cells, and basophils (Carbone *et al.* 1997). CD154 is also secreted in a soluble form, which shows similar activities as the membrane bound form (Graf *et al.* 1995; Mazzei *et al.* 1995).
CD154 is not only the ligand for CD40, it can also act as signal transducing receptor itself. Van Essen *et al.* showed that the interaction between B cells and T cells mediated through CD40 and CD154 is bidirectional, enabling T cells to deliver effective help to B cells and allowing B cells to respond to it (van Essen *et al.* 1995). CD154 signaling pathways are not completely elucidated yet. So far it is known, that CD154 activation leads to phosphorylation of PLCγ and a subsequent calcium release and PKC activation (Brenner *et al.* 1997). This process is abrogated by calcineurin inhibition (Blair *et al.* 2000). Another signal, whose consequences are not clear, conducted by CD154 is the activation of a neutral sphingomyelinase resulting in ceramide production (Koppenhoefer *et al.* 1997). The biological effects of this signaling pathway are not extensively studied. Studies using an approach for activating T cells in the absence of autologous APCs, like DCs or B cells, providing costimulation and cytokines brought some insight, but are still controversial. Cayabyab *et al.* showed that human T cells stimulated with anti-CD3 antibodies and cocultured with a murine cell line expressing human CD40 proliferate, produce IFN-γ and IL-2. These T cells were also able to acquire cytotoxic activity (Cayabyab *et al.* 1994). In similar experiments Blair *et al.* showed that resting human CD4 T cells stimulated with antibodies against CD3 and CD154 proliferate for a short time and secrete IFN-γ, TNF-α, and IL-10 but not IL-2 (Blair *et al.* 2000). These results were confirmed using a murine cell line expressing CD40 instead of an anti-CD154 antibody (Blair *et al.* 2000).

CD40-CD154 blockade in autoimmunity and transplantation

CD40-CD154 crosstalk plays a central role in B and T cell priming and activation. Therefore it is an attractive target to block in different disease models. Especially in autoimmunity and transplantation, promising results have been obtained. An overview over the published antibodies against murine and human CD154 and CD40 is given in tables 1.2 and 1.3.

Table 1.2: Antibodies directed against murine CD154 and CD40

Name	Molecular characteristics	Mechanism	Used in
directed against murine CD154			
MR1	Hamster IgG3	Costimulatory blockade	murine models for tolerance induction in combination with CD28 pathway blockade, DST, bone marrow transplantation
directed against murine CD40			
FGK4.5	Rat IgG2a	activating	e.g. induction of immune responses against tumors (Turner *et al.* 2001)
3/23	Rat IgG2a	activating	
HM40-3	Hamster IgM	activating	

Table 1.3: Antibodies directed against human CD154 and CD40

Name	Molecular characteristics	Mechanism	Used in	Reference
		directed against human CD154		
hu5c8	humanized IgG1	costimulatory blockade, T cell depletion	non-human primate renal transplantation human renal transplantation	(Kirk *et al.* 1999), (Cho *et al.* 2001)
hu24-31, IDEC-131	humanized IgG1	costimulatory blockade	non-human primate skin grafts together with DST and rapamycin, non-human primate renal transplantation together with DST and sirolimus	(Xu *et al.* 2003), (Preston *et al.* 2005)
ABI793	fully human IgG1	costimulatory blockade	non-human primate renal transplantation	(Schuler *et al.* 2004), (Kanmaz *et al.* 2004)
		directed against human CD40		
ch5D12	chimeric IgG4	does not costimulate B cell proliferation, has B cell depleting properties	non-human primate EAE model	(t Hart *et al.* 2005)
Chi220	chimeric IgG1	partial agonistic properties, B cell depleting	non-human primate renal transplantation, non-human primate islet transplantation in combination with CTLA4-Ig	(Pearson *et al.* 2002), (Adams *et al.* 2005)
4D11	human IgG4	costimulatory blockade, B cell depleting	induction and maintenance therapy for non-human primate renal transplantation, phase II clinical trial for renal transplantation	(Aoyagi *et al.* 2009), (Pilat *et al.* 2011)
3A8	mouse IgG2b	costimulatory blockade, still allows sCD154 binding, induces B7 upregulation in B cells	non-human primate islet transplantation, non-human primate renal transplantation together with CTLA4-Ig and sirolimus	(Badell *et al.* 2012), (Page *et al.* 2012)

CD40-CD154 blockade in autoimmunity

Multiple sclerosis is a mainly Th1-mediated autoimmune demyelinating disease of the central nervous system. The CD40-CD154 pathway has been shown to be involved in MS-lesions (Gerritse *et al.* 1996). In models for murine experimental autoimmune encephalomyelitis (EAE), use of a blocking antibody against CD154 could ameliorate disease and even later after treatment prevent disease relapses (Gerritse *et al.* 1996; Howard *et al.* 2002). This was also proven in nonhuman primates: Using a murine antibody blocking CD40 or its chimeric form, EAE could be reduced and even after onset the formation of new lesions could be avoided (Laman *et al.* 2002; t Hart *et al.* 2005). Zheng *et al.* showed reduced autoimmune arthritis in mice treated with an siRNA against CD40 (Zheng *et al.* 2010). Finally, in a murine model for SLE administration of an anti-CD154 antibody early in life could delay onset of disease (Mohan *et al.* 1995). And when combining CD154- with CD28-blockade in this setting, onset of lupus nephritis could be delayed for months (Daikh *et al.* 1997).

CD40-CD154 blockade as immunosuppression in transplantation

The importance of the CD40-CD154 pathway has also been studied in alloreactivity: CD154 deficient T cells fail to induce graft-versus-host disease, when injected into F1 recipients (Buhlmann and Noelle 1996). Furthermore, the rejection of allogeneic cells injected into CD154 knock-out animals is impaired (Shepherd and Kerkvliet 1999). CD154 deficient recipients of cardiac allografts show long term allograft survival and donor-specific tolerance (Shimizu *et al.* 2000). Finally, the CD40 pathway has been shown to be more critical for induction of cardiac allograft rejection than the CD28 pathway (Bingaman *et al.* 2001). Despite the beneficial effect of CD154 deficiency on acute rejection, chronic rejection still occurs (Shimizu *et al.* 2000).
However, blockade of the CD40-pathway alone is not sufficient to induce stable tolerance to allografts. The survival of cardiac or islet allografts is prolonged, when CD40 is blocked at the time of transplantation (Larsen *et al.* 1996; Parker *et al.* 1995). But eventually all allografts in these studies were rejected. However, if CD40-CD154 blockade is combined with the blockade of the CD28 costimulatory pathway, murine cardiac allografts survive long term without signs of rejection (Larsen *et al.* 1996). Interestingly, addition of cyclosporin A to this protocol prevents allospecific tolerance, indicating that T cells need to receive signal 1 via the TCR in order to be tolerized (Larsen *et al.* 1996). This mechanism has been shown to be true also for the induction of anergy in human T cells *in vitro* (Koenen *et al.* 2005). The combination of blockade of both costimulatory pathways (CD40 and CD28) was successfully translated into non-human primate models for renal and islet transplantation (Adams *et al.* 2005; Kirk *et al.* 1997).

Tolerance induction by donor-specific transfusion

Several strategies to induce donor-specific tolerance are under investigation in murine and non-human primate models. Most of them rely on blockade of the CD40-CD154 pathway. One strategy to induce donor specific tolerance is the combination of donor-specific transfusion (DST) in combination with CD40-CD154 pathway blockade by antibodies. DST can consist of donor blood or splenocytes (Markees *et al.* 1998; Zheng *et al.* 1999). This strategy has been shown to efficiently prolong allograft survival in murine models for islet, heart and airway allografts (Chalermskulrat *et al.* 2006; Zheng *et al.* 1999). For long term skin graft survival DST under CD154 blockade is only sufficient, when the recipients are thymectomized (Markees *et al.* 1998; Phillips *et al.* 2003). Mechanistical studies have shown that both T cell subsets (CD8 and CD4) contribute to allospecific tolerance induced in this way and that especially indirectly alloreactive T cells are rendered hyporesponsive (Gao *et al.* 2004; Kishimoto *et al.* 2004; Phillips *et al.* 2006). Furthermore, a CD4 dependent deletion of directly alloreactive CD8 T cells has been observed (Iwakoshi *et al.* 2000). Finally, a similar protocol showed efficacy in preventing renal allograft rejection in a non-human primate model. Recipients were treated with DST under CD154 blockade (for eight weeks) combined with an mTOR-inhibitor (sirolimus, for three months). Three out of five animals receiving the triple therapy survived rejection-free for more than 500 days after therapy withdrawal and remained tolerant even after a secondary donor-specific skin graft challenge (Preston *et al.* 2005). Interestingly, TLR-signaling prevents tolerance induction achieved by CD40-CD154 blockade and DST. When TLR agonists are administered simultaneously with the tolerogenic treatment, B7-molecles are upregulated on transfused APCs and no tolerance is achieved (Thornley *et al.* 2006).

Tolerance induction by mixed chimerism

Another way to achieve donor-specific tolerance is the induction of mixed chimerism. The basis for this is the observation made by Owen in dizygotic bovine twins, in which due to anastamoses of placental vessels both individuals had red blood cells from the other circulating in the bloodstream (Owen 1945). These animals were tolerant to skin grafts from the respective twin but not to those from siblings of separate birth (Billingham and Reynolds 1952). This state was thought to be due to the permanent engraftment of precursor cells that were exchanged during fetal development, a time at which the immature immune system is not capable of rejection yet. This could be proven by performing in utero transplantation of donor-type cell mixtures in chicken, rabbits, and mice (Billingham *et al.* 1953; Billingham *et al.* 1955). Thus, the principle of tolerance achieved by mixed chimerism differs from the strategies mentioned above. In this case tolerance relies on a central deletion of alloreactive T cells by donor bone marrow derived DCs that engraft in the thymus. This principle was translated to an induction of mixed hematopoetic chimerism in adult animals with an established immune system applying a myeloablative irradiation and

subsequent host and donor-derived bone marrow transplantation (Main and Prehn 1955; Ildstad and Sachs 1984). The protocols have been improved since then. The conditioning was reduced to a non-myeloablative, sparing enough of the host bone marrow to allow survival also without subsequent bone marrow transplantation (Sharabi *et al.* 1992). Now it is known that in mice a minimal treatment including 3 Gy total body irradiation and a single dose of a blocking anti-CD154 antibody is sufficient to induce a stable mixed chimerism (Fehr *et al.* 2005; Takeuchi *et al.* 2004; Wekerle *et al.* 1999). Induction of mixed chimerism has been shown to lead to indefinite survival of skin, islet, lung, or cardiac allografts in murine models (Guo *et al.* 2008; Li *et al.* 2008; Seung *et al.* 2000). A similar protocol could also be translated to non-human primates and long term renal allograft tolerance was achieved in five out of eight recipients (Kawai *et al.* 2004). Also a small series of patients underwent treatment for mixed chimersim induction combined with renal transplantation. Four of five patients accepted their kidney allograft (Kawai *et al.* 2008).

DNAX accessory molecule-1 and its ligands

DNAX accessory molecule-1

DNAX accessory molecule-1 (DNAM-1, CD226) was first described in 1996 as a novel adhesion molecule involved in the cytolytic function of T lymphocytes (Shibuya *et al.* 1996). DNAM-1 belongs to the immunoglobulin superfamily and its sequence shows similarity with CD96 (TACTILE) (Shibuya *et al.* 1996). DNAM-1 is expressed on all T cell subsets including $\gamma\delta$T cells, NK cells, monocytes and on a subset of B cells (Shibuya *et al.* 1996). Later DNAM-1 was shown to be identical to a membrane protein described earlier on platelets and called PTA1 (Kojima *et al.* 2003; Scott *et al.* 1989).
DNAM-1 plays a role in several processes in the immune system, which are described in the following paragraphs:

1. T cell priming
2. Cytotoxic T cell activity against tumor cells and non-professional APCs
3. Cytotoxic activity of NK cells against tumor cells and DCs
4. Extravasation of monocytes across the endothelial barrier
5. Platelet adhesion to endothelial cells

The accessory molecule lymphocyte function-associated antigen 1 (LFA-1) has been shown to not only be important for lymphocyte adhesion, but also during priming of naive T cells. The interaction of LFA-1 and its ligand ICAM-1 (CD54) expressed on the antigen presenting cell allows to keep the cell-cell contact long enough for the T cell to recognize its cognate antigen. If the TCR binds to a matching MHC-peptide complex, LFA-1 affinity to ICAM-1 is increased to further prolong T cell-APC contact. It has been shown, that the activation of naive human T cells with an antibody against CD3 and one activating LFA-1 leads to T cell proliferation and Th1 induction (Shibuya *et al.*

2003). The phosphorylation of DNAM-1 at tyrosine 322 is essential for this process (Shibuya *et al.* 2003).
DNAM-1 on NK and cytoxic T lymphocytes (CTLs) also plays a role in rejection of tumor cells expressing DNAM-1 ligands as shown in DNAM-1 deficient mice (Gilfillan *et al.* 2008; Iguchi-Manaka *et al.* 2008). Apart from this defect DNAM-1 deficient mice show a normal composition of lymphocyte populations in the spleen, bone marrow, and lymph nodes (Gilfillan *et al.* 2008; Iguchi-Manaka *et al.* 2008). Ectopic expression of DNAM-1 ligands can increase NK mediated cytotoxicity against tumor cell lines (Tahara-Hanaoka *et al.* 2004). The same is true for CD8 T cell proliferation in response to non-professional APCs like B cells pulsed with antigen (Gilfillan *et al.* 2008). Whereas the activation of CD8 T cells by professional APCs does not seem to be dependent on DNAM-1 (Gilfillan *et al.* 2008), cytolytic activity of NK cell against DCs is (Pende *et al.* 2006).
DNAM-1 has two known ligands: CD155 (Necl-5, Tage4, PVR) and CD112 (Nectin-2, Prr2, HVEB) (see Fig.1.5) (Bottino *et al.* 2003; Tahara-Hanaoka *et al.* 2004). Both molecules have been shown to be expressed on endothelial cells (Reymond *et al.* 2004). The interaction between CD155 and DNAM-1 is crucial for the transmigration of human monocytes across an endothelial monolayer, as the process of trans-endothelial migration is blocked in the diapedesis phase when DNAM-1 is blocked (Reymond *et al.* 2004). Furthermore, the activation of human CD8 T cells by endothelial cells has been shown to be partially influenced by DNAM-1/CD155 interaction. Interestingly, CD8 T cells proliferate similarly but produce more IFN-γ, when CD155 or DNAM-1 is blocked (Escalante *et al.* 2011). Expression of CD155 on endothelial cells has been shown to be important for platelet adhesion to endothelial cells. Thrombin-activated platelets show phosphorylation of DNAM-1 at tyrosine 322 and blockade of DNAM-1 with an antibody reduces adhesion of platelets to human endothelial cells (Kojima *et al.* 2003). Finally, blockade of DNAM-1 leads to amelioration of CD8 T cell mediated graft-versus-host-disease in a murine model (Nabekura *et al.* 2010).

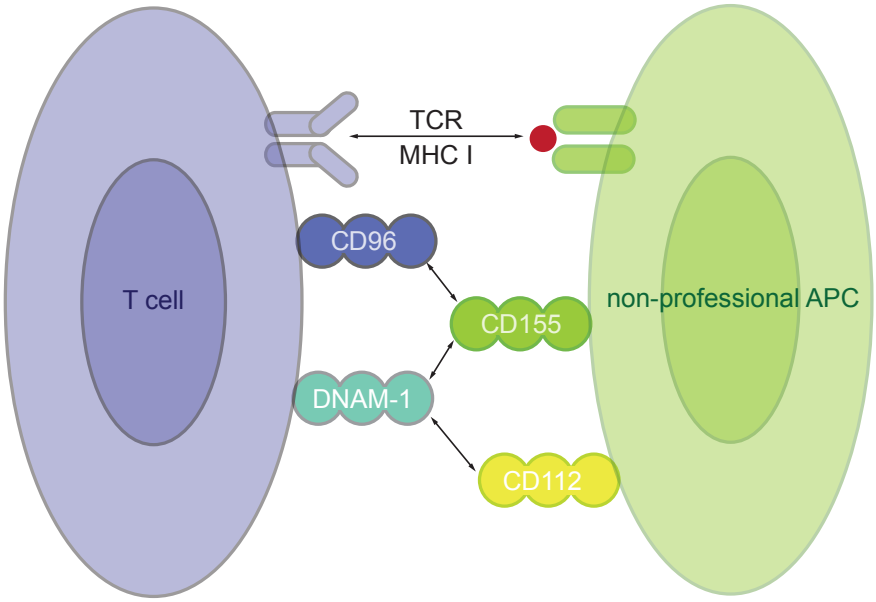

Figure 1.5: Schematic illustration of the interactions between a T cell and a non-professional APC. The T cell recognizes its cognate antigen in the context of MHC with its T cell receptor (TCR). For further activation the T cell needs further costimulatory signals, which can be delivered via DNAM-1 ligation by its two ligands CD155 and CD112 expressed on the non-professional APC. CD155 has another receptor called CD96.

DNAM-1 ligands

The two ligands for DNAM-1, CD112 (nectin-2) and CD155 (necl-5), belong to the nectin- and nectin-like (necl) family of cell adhesion molecules, respectively. The family consists of nectin-1 to -4 as well as necl-1 to -5. These molecules are immunoglobulin-like adhesion molecules, with three immunoglobulin-like loops in the extracellular region, a transmembrane segment and a cytoplasmic tail. Nectin or necl proteins are able to bind nectins of the same or different types on the adjacent cell surface after forming a homodimer in cis. In general heterophilic trans-interactions are stronger then homophilic trans-interactions (Satoh-Horikawa *et al.* 2000). Nectins initiate the formation of adherence junctions between epithelial, endothelial, or fibroblast cells, before cadherins are involved (Takai *et al.* 2003).
CD112 acts as entry-receptor for α-herpesvirus and is expressed on endothelial and epithelial cells in various organs, in mouse especially in liver and kidney (Bottino *et al.* 2003; Nabekura *et al.* 2010; Takai *et al.* 2008). CD112 expression has also been shown to be pronounced on cells lining high endothelial venules in human lymph nodes (Pende *et al.* 2006). Furthermore, expression on human monocyte-derived DCs has been shown (Pende *et al.* 2006). CD112 knock-out mice show no obvious phenotype apart from male infertility due to defects in nuclear and cytoskeletal morphology and mitochondrial localization in spermatozoa (Bouchard *et al.* 2000; Mueller *et al.* 2003).

The second ligand for DNAM-1, CD155, has a higher affinity to DNAM-1 than CD112 (Reymond *et al.* 2004; Seth *et al.* 2009). CD155 acts as receptor for poliovirus (Takai *et al.* 2008). It is also expressed on various epithelial and endothelial cell types and APCs (Gilfillan *et al.* 2008). In the mouse it has been localized mostly to liver, heart, and kidney (Nabekura *et al.* 2010). CD155 has been shown to play a major role in cell movement and is essential for the formation of leading-edge structures (Takai *et al.* 2008). Furthermore, endocytosis of CD155 upon newly formed cell-cell contact after movement leads to a signaling pathway that inhibits further movement and proliferation (Takai *et al.* 2008). CD155 is upregulated in different types of cancer cells, and this is correlated with enhanced metastasis (Ikeda *et al.* 2004; Morimoto *et al.* 2008). In contrast to most of the other nectin and necl molecules CD155 is not able to build homodimers in trans (Takai *et al.* 2008). CD155 knock-out mice do not show an overt phenotype and display normal cell distribution in most lymphatic organs (Maier *et al.* 2007). However, these mice show a reduced number of CD8 single positive T cells in the thymus and a reduced ability to mount humoral immune responses to orally administered antigens (Maier *et al.* 2007; Qiu *et al.* 2010).
CD155 binds to a second receptor CD96 (tactile), which is expressed on T and NK cells but not on the majority of B cells, monocytes and granulocytes (Meyer *et al.* 2009). Their interaction has been shown to promote NK mediated killing of tumor cells (Fuchs *et al.* 2004). CD96 is upregulated on T cells late after activation (six to nine days) (Meyer *et al.* 2009; Wang *et al.* 1992). CD96 expression has furthermore been found in a variety of adult human tissues (Meyer *et al.* 2009). Disruption of the CD96 gene has been linked to manifestation of the C syndrome (Opitz Trigonocephaly), indicating a role for CD96 in developmental processes in addition to its immunologic function (Kaname *et al.* 2007).

Renal tubular epithelial cells as non-professional APCs

Renal tubular epithelial cells (rTECs) are the cells lining the tubulus. They have the important task to reabsorb water and ions from and secrete waste products into the primary urine formed in the glomerulus. Thereby they maintain mineral ion homeostasis and water balance. Apart from that rTECs also execute an immunomodulatory function by expression of certain surface molecules and secretion of chemo- and cytokines. Under inflammatory conditions rTECs upregulate MHC class I and II molecules (Wuthrich *et al.* 1990). They also express the costimulatory molecules CD40 and ICOS-L (Starke *et al.* 2007). However, the two ligands for the major costimulatory receptor CD28 B7-1 and B7-2 are not expressed on rTECs (Waeckerle-Men *et al.* 2007). On the other hand rTECs are able to express the coinhibitory molecule PD-L1 and thereby regulate T cell activity (Starke *et al.* 2010; Waeckerle-Men *et al.* 2007). Upon inflammatory stimulation rTECs themselves secrete cytokines and chemokines. Amongst these are IL-6, IL-8, RANTES, MCP-1, IL-15 and PAI-1 (Woltman *et al.* 2000). These proinflammatory messengers help recruiting immune effector

cells to the site of injury and enhance their function. This may help to clear pathogens in the kidney but can cause further tubular injury during allergic interstitial nephritis and accelerate renal allograft rejection (Dugger *et al.* 2009). Thus, during renal allograft rejection, not only the interaction between professional APCs and T or B cells has to be taken into account. Also, the interaction between effector cells and the major target cells is highly regulated due to the properties of rTECs.

Objective of this study

Costimulation is an important process during T and B cell activation. It is known, that blockade of certain costimulatory pathways in combination or alone is beneficial to prevent allograft rejection (Pilat *et al.* 2011). This work was designed to investigate some of the costimulatory processes taking place during renal allograft rejection.
Blockade of the CD40-CD154 pathway is able to prevent acute allograft rejection (Quezada *et al.* 2004), which is mainly mediated by directly alloreactive T cells (Benichou *et al.* 1999). We therefore sought to explore the role of CD40 expressed on donor cells in renal allograft rejection. Hereby we not only focused on CD40 on donor derived APCs, which induce a direct alloresponse. As tubular epithelial cells are able to express CD40 under certain conditions (Starke *et al.* 2007), we also investigated the role of CD40 on them *in vitro* and *in vivo*.
The research on CD40-CD154 blockade in murine models so far relies mainly on a blocking antibody against CD154 (MR1). However, the translation of CD154 blockade into clinics has led to intolerable side effects and is thus not pursued anymore. It is thus necessary to find tools to block the receptor for CD154 in order to determine, whether CD40 blockade is as efficient as CD154 blockade in the well established murine models for tolerance induction and allograft rejection. As there is so far no anti-CD40 antibody without at least partially agonistic features, we sought to develop a F(ab)-fragment directed against CD40 with exclusively antagonistic properties. We chose to use a monovalent F(ab)-fragment in order to avoid cross-linkage of the CD40 receptor, which might lead to activation.
Finally, we investigated the role of the novel costimulatory molecule DNAM-1. It has been shown, that DNAM-1 ligation is crucial for the cytotoxic activity of CTLs against non-professional APCs (Gilfillan *et al.* 2008). As rTECs can act as non-professional APCs under inflammatory conditions and are the major targets of CTLs during interstitial rejection, we analyzed, if there is a relevance of the DNAM-1 pathway in the process of renal allograft rejection.

Chapter 2

Results

Section 1
Absence of donor CD40 inhibits allospecific CD8 and Th17 responses and protects renal allograft epithelium

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All experiments, except renal allografts, histology, and immunohistochemistry were performed by Anna K. Kraus. The manuscript was written and the figures were made by Anna K. Kraus.

Abstract

Blocking the CD40-CD154 pathway is efficient in various experimental models to prevent allograft rejection and induce donor-specific tolerance. However, the translation to clinical studies has been hampered by unexpected thromboembolic complications of CD154-blocking antibodies. Thus, blocking CD40 instead is now considered as an alternative strategy. Here we evaluated the role of donor CD40 in allospecific T cell responses *in vitro* and *in vivo*. Absence of donor CD40 reduced all effector functions of allospecific T cells *in vitro*. Donor CD40 expression was essential for the induction of allospecific Th17 cells. Interestingly, CD8 T cells required direct CD154 ligation by allogeneic dendritic cells in order to become fully activated, even in the presence of functional CD4 help. As a result absence of CD40 led to prolonged survival of MHC class I-mismatched skin grafts. Furthermore, fully MHC mismatched renal allografts from CD40 knock-out (KO) donors displayed better renal function despite histological findings comparable to wild type allografts. These functional data correlated with a lower level of apoptosis in renal tubular cells and higher expression of PD-L1. In conclusion, CD40 blockade not only reduces the allospecific T cell responses, but may also lead to protection of tubular epithelium from apoptosis and thereby preserve kidney allograft function.

Introduction

Kidney transplantation is the preferred therapy for end stage renal disease (Wolfe *et al.* 1999). Despite improvements in early graft survival, chronic rejection and toxicity of classical immunosuppressant drugs remain major reasons for late allograft loss (Hariharan *et al.* 2000). Thus, there is a need for more specific and less toxic immunosuppression regimens. Blockade of the CD40-CD154-pathway represents a promising target to achieve this goal. It has been shown that blockade of CD154 together with blockade of B7-molecules (CD80 and CD86) lead to long-term graft survival in murine and non-human primate models of skin and renal allografts (Kirk *et al.* 1997; Larsen *et al.* 1996). Furthermore CD154 blockade was used in several protocols for tolerance induction using donor specific transfusion (Markees *et al.* 1997; Rossini *et al.* 1996) or bone marrow transplantation (Fehr *et al.* 2005; Takeuchi *et al.* 2004; Wekerle *et al.* 1999). Many murine protocols for tolerance induction used a monoclonal antibody directed against CD154 (MR1). However, translation of the anti-CD154 reagent into non-human primate and human studies revealed an unexpected complication of thromboembolic events (Kawai *et al.* 2000; Knechtle *et al.* 2001), which were due to platelet activation (Koyama *et al.* 2004). Thus, CD40 instead of its ligand turned into an attractive target (Badell *et al.* 2012 ; Page *et al.* 2012).
Renal tubular epithelial cells (rTECs) are the main target of alloreactive T cells during tubulointerstitial rejection of renal allografts (Cornell *et al.* 2008). RTECs function as non-professional antigen presenting cells (APCs). They express MHC class I and II molecules under inflammatory conditions (Wuthrich *et al.* 1990). The only classical costimulatory molecule expressed on activated rTECs is CD40 (Waeckerle-Men *et al.* 2007), whereas CD80 and CD86 are not. With this surface expression pattern rTECs become targets for both alloreactive CD8 and CD4 T cells.
The T helper cell population mainly involved in acute rejection is of a Th1 type characterized by IFN -γ production (Benichou *et al.* 1999). However, a new subset of pro-inflammatory Th cells, namely Th17 cells, has come into the focus of transplant research (Heidt *et al.* 2010). They are characterized by the expression of the proinflammatory cytokines IL-17A, IL-17F, IL-21 and IL-22 (Ouyang *et al.* 2008). This T cell subtype is important for the clearance of pathogens, which cannot be adequately cleared by Th1 or Th2 cells, and it has been described to play a role in various autoimmune diseases (Crome *et al.* 2009). IL-17 has been detected in early renal allograft rejection in rats and humans (Loong *et al.* 2002). Furthermore, human rTECs stimulated with IL-17 become activated and in turn start to secrete cytokines like IL-8 and IL-6 (Woltman *et al.* 2000). Nevertheless, the exact role of Th17 cells in allograft rejection is not clear.
In this study we investigated the role of donor CD40 during direct alloreactivity *in vitro* and *in vivo*. We demonstrate an important role of donor CD40 expression for the induction of directly alloreactive cytotoxic T lymphocytes and Th17 cells *in vitro*. Absence of donor CD40 prolonged MHC class I-mismatched skin grafts, reduced tubular cell apoptosis and improved kidney function in completely MHC-mismatched renal allografts.

Material and Methods

Mice

Wild type (WT) C57BL/6 (B6, H-2^b), CD40 knock-out (KO) (B6 background, H-2^b), CD154 KO (B6 background, H-2^b), CBA (H-2^k), Balb/c (H-2^d), and B6.C-H2-K^{bm1}/By (bm1, H-2^{bm1}) mice were housed in specific pathogen-free conditions at the University of Zürich. Bm1 mice express the same H-2 haplotype as B6 (H-2^b) except for 7 nucleotide differences in the gene for H-2K^b resulting in amino acid substitutions at codons 152 (glutamate to alanine), 155 (arginine to tyrosine) and 156 (leucine to tyrosine) (Schulze *et al.* 1983). IL-17A KO mice (B6 background, H-2^b) were kindly provided by Burkhard Becher with the permission of Yoichiro Iwakura (Nakae *et al.* 2002). All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office of the Canton of Zürich).

rTEC culture

Preparation and primary culture of rTECs were performed as described (Wuthrich *et al.* 1990). In all cytotoxicity experiments primary rTEC targets were stimulated for 48 hours with murine IFN-β and -γ (both Antigenix America, Huntington Station, NY, USA) at 100 U/ml each prior to use. For FACS analysis of surface marker expression rTECs were stimulated with indicated concentrations of IFN-γ, IL-17A (Antigenix America, Huntignton Station, NY USA) or an agonistic anti-CD40 monoclonal antibody (mAb) (FGK 4.5, BioXCell, West Lebanon, NH, USA).

T cell proliferation and cell-mediated lympholysis (CML) assay

T cell proliferation and CML assays were performed using isolated CD4 and CD8 positive T cells as responders. T cells were stimulated with irradiated (30 Gy) splenocytes, CD11c positive DCs or splenocytes depleted from CD11c positive cells from allogeneic and syngeneic mice. Splenocytes were sorted by magnetic cell separation (MACS) according to the protocols of Miltenyi Biotec (Bergisch Gladbach, Germany). Purity of sorted cells was confirmed by fluorescence-activated cell sorting (FACS) analysis. Purity for T cells was usually $> 90\%$ and for DCs $> 50\%$.
T cell proliferation was measured by incorporation of tritium-labeled thymidine (Perkin Elmer, Waltham, USA) on day (d) 4 of culture. Stimulation indices were calculated as follows:

$$\text{Stimulation index} = \frac{\text{incorporation allogeneic sample}}{\text{mean incorporation of syngeneic controls}}$$

CML assays were performed on d 5 of culture: ^{51}Chromium (Cr)-labelled, IFN-stimulated allogeneic rTECs were added to the serially diluted culture for 4 hours (killing phase), and allospecific cytotoxicity was assessed by measurement of ^{51}Cr release in the supernatant. Allospecific lysis was calculated as:

$$\%\ \text{specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} * 100$$

FACS analysis

FACS analysis was performed on a BD-FACSCanto II (Becton Dickinson, Allschwil, Switzerland). Anti-mouse CD3-FITC, CD4-PE, CD8-APC, CD40-APC, PD-L1 mAb, secondary anti-Rat FITC, and propidium iodide (PI) were purchased from eBioscience (Frankfurt, Germany).
Cytokines in cell culture supernatants were quantified using a FlowCytomix set purchased from eBioscience (Frankfurt, Germany) according to the manufacturer's manual.

mRNA isolation and quantitative PCR (qPCR)

mRNA was isolated from either kidney grafts or naive kidneys stored in RNase-inhibitor or freshly sorted cells using the RNeasy Mini Kit (Qiagen, Hombrechtikon, Switzerland). One μg mRNA was transcribed to cDNA using the Omniscript reverse transcription Kit (Qiagen). Pre-developed TaqMan reagents were used for qPCR (Applied Biosystems, Carlsbad, CA, USA) detecting murine PD-L1, IFN-γ, IL-17A, and the reference 18s rRNA. The expression of candidate genes was normalized to the reference, and fold changes were calculated in relation to the matching controls unsing the 2^{-ddCT} method.

Skin and kidney grafting

At d 0 full thickness tail skin (about 0.5-1.0 cm^2) from donor mice was transplanted to the dorsal flank area of recipient mice. Graft rejection was defined as graft necrosis $> 90\%$ of the graft.
Kidney grafts were performed in a life-supporting manner as described in detail previously (Tian *et al.* 2010). In brief, CBA recipients were bilaterally nephrectomized and only the right kidney was replaced by a B6 WT or CD40 KO allograft. In this strain combination we performed up to today about 20 allografts and no incidence of spontaneous tolerance was observed. Recipients were sacrificed one week after transplantation. In order to assess graft function, plasma urea concentration was measured with the urease/glutamate dehydrogenase method on a Hitachi Modular P autoanalyzer from Roche diagnostics (Rotkreuz, Switzerland).

Histology and immunohistochemistry

Histologic examination of all kidney grafts was performed by an experienced renal pathologist blinded to the experimental procedures. Tissues were immersion-fixed in 4% phosphate buffered formalin and embedded in paraffin. The thickness of sections was 4 μm. The slides were routinely stained with hematoxylin and eosin (H & E), periodic acid-Schiff stain (PAS) and Elastica-van Gieson (EvG). In selected cases silver methenamine stain and acid fuchsin orange-G stain were added.

Immunohistochemistry for CD3 was performed on paraffin embedded material as previously described using a monoclonal rat anti-CD3 anitbody (Clone CD3-12, AbD serotec, Dusseldorf, Germany) (Vielhauer *et al.* 2009). For detection of apoptotic cells by immunohistochemistry the monoclonal antibody F7-26 (Chemicon, International, Inc. Temecula, CA) was used as previously described (Segerer *et al.* 2002). F7-26 binds to single-stranded DNA after thermal denaturation. A peroxidase-conjugated monoclonal rat anti-mouse IgM antibody (Zymed, San Francisco, CA) was used as secondary reagent. Dense, apoptotic nuclei positive for single stranded DNA were quantified in mouse renal allografts in 15 high power fields (original magnification $\times 250$).

Statistical analysis

All statistical comparisons were performed with GraphPad Prism 4. Normally distributed groups were compared using Student's t test. Groups without Gaussian distribution were compared using the Mann-Whitney test. $P < 0.05$ was considered as significant.

Results

Effector functions of directly alloreactive T cells and Th17 induction depend on donor CD40

To assess direct alloresponses *in vitro* we cocultured isolated T cells from CBA mice (H-2^k) with irradiated splenocytes from WT B6 or CD40 KO (both H-2^b) donors. Proliferation was reduced, when the stimulator cells did not express CD40 (Fig. 2.1A). To measure allospecific cytotoxicity CBA T cells were stimulated as described above and then used in a Cr-release assay using IFN-treated rTECs as targets. Allospecific cytotoxicity was reduced, when the T cells did not receive a CD40 signal during stimulation (Fig. 2.1B). CD40 on target rTECs, however, did not influence cytotoxicity (data not shown).
In order to characterize the subtype of allospecific T helper cells generated in the cocultures described above, we measured cytokine production in the supernatant by multiplex bead assay. A strong induction of IFN-γ production was detected in cocultures of CBA T cells with WT B6 stimulators, which was markedly reduced in the absence of CD40 (Fig. 2.1C). Furthermore, we detected production of IL-6 and subsequently IL-17A. The production of IL-17A was restricted to CD4 T cells as assessed by ELISA and qPCR (data not shown) and was strongly inhibited in the absence of donor CD40 (Fig. 2.1D). Th2 cytokines such as IL-4 and IL-10 were not found in any coculture (data not shown).
To analyze whether there is coherence between Th17 induction and cytotoxic activity, we stimulated B6 IL-17A KO CD4 and WT CD8 T cells with completely MHC-mismatched Balb/c splenocytes and measured proliferation and cytotoxic activity. Neither proliferation nor cytotoxic activity was affected by the ability of CD4 T cells to secrete IL-17A (Fig. 2.1E, F).

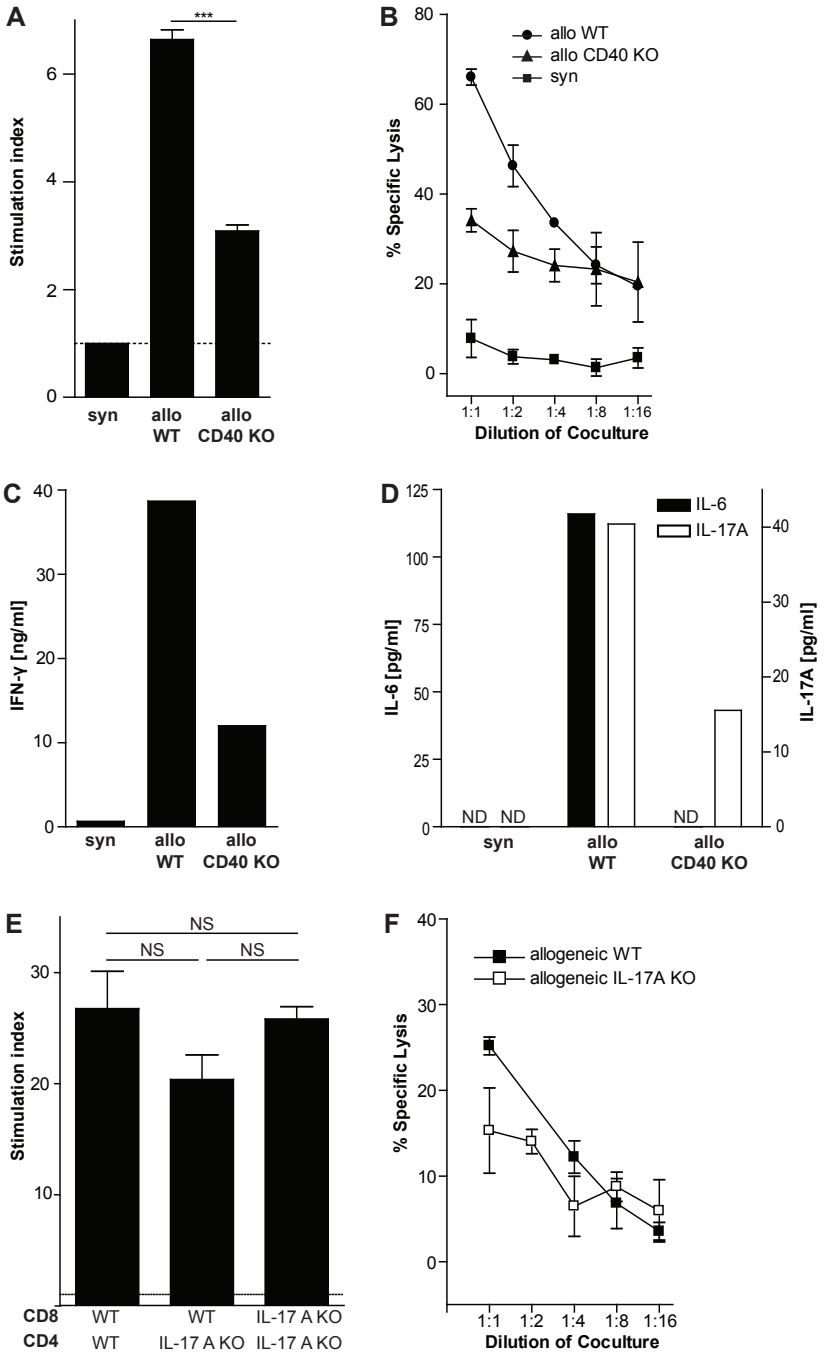

Figure 2.1: Directly alloreactive T cells depend on donor CD40. CBA CD4 and CD8 T cells were isolated by MACS and cultured in a ratio of 2:1. T cells were stimulated with irradiated allogeneic B6 WT or CD40 KO splenocytes. (A) Allospecific T cell proliferation was measured on d 4 of culture. $***P < 0.0001$ (B) Allospecific cytotoxicity was measured on d 5 in a Cr-release assay against IFN-stimulated rTECs from WT B6 mice. (C, D) Cytokine content of coculture supernatants was measured on d 4 using a FlowCytomix assay: (C) IFN-γ, (D) IL-6 and IL-17A concentrations. (E, F) B6 WT and IL-17A KO CD4 and CD8 T cells were isolated by MACS and cultured in a ratio of 2:1. T cells were stimulated with irradiated allogeneic Balb/c splenocytes. (E) Allospecific proliferation of the indicated T cell combinations was measured on d 4 of culture. (F) Allospecific cytotoxicity was measured on d 5 in a Cr-release assay against IFN-stimulated rTECs from Balb/c mice. Representative data of at least 2 independent experiments are shown

Combined Th17 and Th1 responses induce a costimulatory state of rTECs *in vitro*

rTECs respond to the cytokine milieu surrounding them. When stimulated with IFN-γ they upregulate MHC molecules, CD40 but also the coinhibitory molecule PD-L1 (Starke *et al.* 2007). PD-L1 expression can also be induced by CD40 activation on human primary rTECs (Chen *et al.* 2006).
As we detected a strong effect on Th1 and Th17 responses, when stimulating APCs did not express CD40, we assessed the effect of IFN-γ and IL-17A on the surface expression pattern of rTECs. When rTECs were stimulated with IL-17A alone, no alteration of surface expression was detected (data not shown). Treatment of rTECs with IL-17A in combination with IFN-γ, reduced the expression of PD-L1 on WT and CD40 KO cells compared to the treatment with IFN-γ alone (Fig. 2.2A). This reduction was not reversed by stimulating the CD40 pathway with FGK 4.5 on WT rTECs (Fig. 2.2A). Furthermore, the expression of CD40 on WT rTECs is induced upon IL-17A addition and was further increased, when the CD40-pathway is activated (Fig. 2.2B). Thus, a Th17 response with a concomitant Th1 response renders the surface expression pattern of rTECs to a rather costimulatory than coinhibitory state.

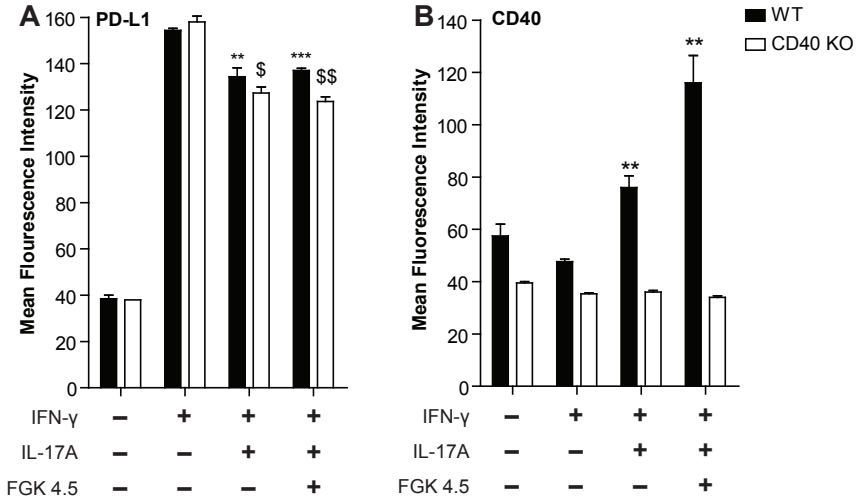

Figure 2.2: Th1 and Th17 responses together induce a costimulatory state of rTECs *in vitro*. WT B6 and CD40 KO rTECs were cultured with the indicated stimuli for 48 hours (IFN-γ at 100 U/ml, IL-17A at 50 ng/ml, anti-CD40 mAb FGK 4.5 at 20 μg/ml). Surface expression of PD-L1 (A) and CD40 (B) was assessed by FACS. Mean fluorescence intensities of triplicates are presented. $^{**}P < 0.008$, $^{***}P < 0.0003$ compared to WT cells stimulated with IFN-γ alone. \$ $P < 0.001$, \$\$ $P < 0.0005$ compared to CD40 KO cells stimulated with IFN-γ alone. Representative results of three independent experiments are shown.

CD4 help and donor CD40 are crucial for induction of directly alloreactive CD8 T cells

CD4 T cells provide help to CD8 CTLs by producing stimulatory cytokines (e.g. IL-2, IFN-γ), but also by licensing DCs to provide proper costimulation to CD8 T cells. This process has been shown to be critically dependent on CD40-CD154 interaction (Bennett *et al.* 1998; Schoenberger *et al.* 1998). Consistent with these studies, significantly lower proliferation was observed, when CD8 T cells were allospecifically stimulated in the absence of CD4 T cells (Fig. 2.3A), and cytotoxicity against allogeneic rTECs was completely abolished without T help (Fig. 2.3B). Importantly, also the remaining proliferation of "helpless" CD8 T cells was critically dependent on CD40 expressed on donor APCs (Fig. 2.3C). We therefore suspected that CD8 T cells received a CD40 signal directly from the APC. To address this question more precisely, we used B6 (H-2^b) CD154 KO T cells and stimulated them with completely MHC mismatched WT Balb/c splenocytes. When cultured alone, both T cell subsets (CD4 and CD8) showed reduced proliferation in response to alloantigen, if they were not able to express CD154 (data not shown). When combining WT or CD154 KO CD8 with CD154 KO CD4 T cells, allospecific proliferation was reduced - most probably due to impaired CD4 help. However, also the abrogation of CD154 signaling only in CD8 T cells led to reduced overall proliferation. A direct interaction between CD40 on CD4 and CD154 on CD8 T cells could be excluded with a control combining WT CD8 and CD40 KO CD4 T cells (Fig. 2.3D). Thus, this experiment revealed a direct role of CD40 on allo-APCs for CD8 proliferation, even in the presence of a functional CD4 compartment.

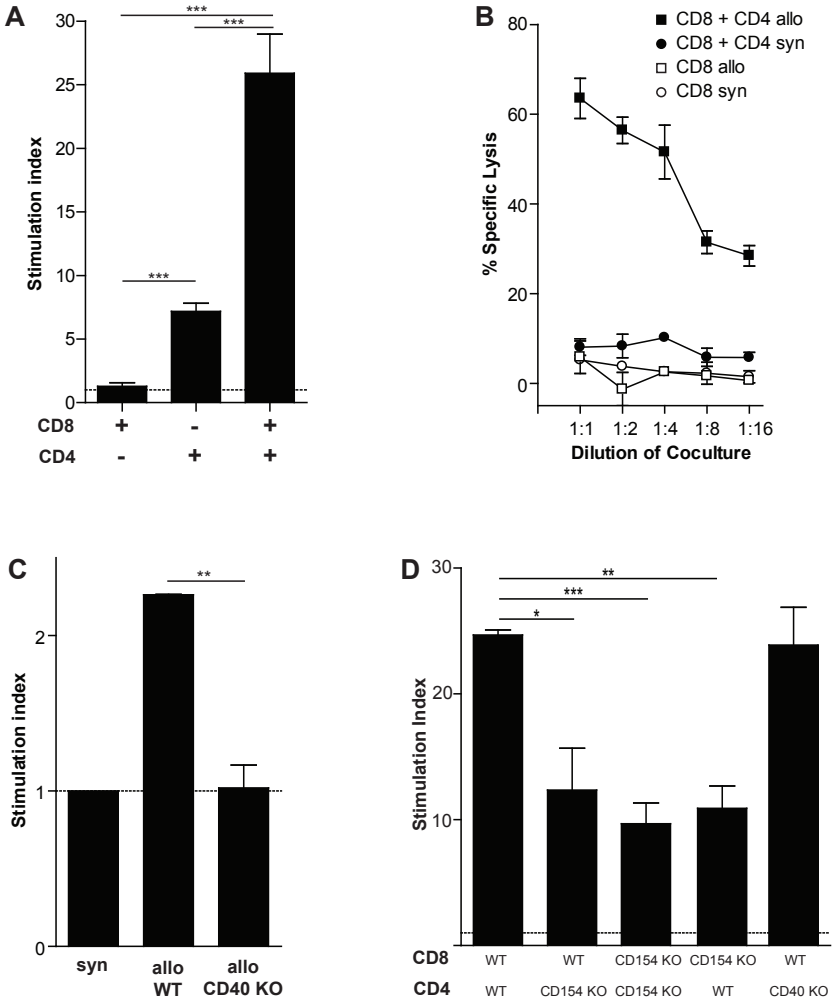

Figure 2.3: CD4 help and CD40 on the APC are crucial for direct allospecific CD8 T cell activity. (A) MACS-sorted CBA T cells were stimulated with irradiated B6 WT splenocytes in the indicated combinations. Proliferation was measured on d 4. (B) CBA CD8 T cells were stimulated with irradiated B6 WT splenocytes in the presence or absence of syngeneic CD4 T cells (ratio of CD4:CD8 being 2:1). On d 5 of culture a Cr-release assay against IFN-stimulated rTECs from B6 WT mice was performed. Filled symbols: CD8 T cells in presence of CD4 help; open symbols: CD8 T cells alone. (C) CBA CD8 T cells were MACS sorted and cocultured with irradiated B6 or CD40 KO splenocytes. Proliferation was measured on d 4 of culture. (D) B6 WT or CD154 KO CD4 and CD8 T cells were isolated by MACS and cultured in a ratio of 2:1. T cells were stimulated with irradiated Balb/c splenocytes. Allospecific T cell proliferation was measured on d 4 of culture. $^{*}P < 0.05$, $^{**}P < 0.005$, $^{***}P < 0.001$. Representative data of at least 3 independent experiments are shown.

CD40 on donor DCs is critical for cytotoxic T cell responses *in vitro*

CD40 is expressed on APCs such as DCs and B cells, two of the major APC-populations. Using isolated DCs from B6 and CD40 KO mice as stimulators, allospecific proliferation and cytotoxicity of CBA T cells were induced (Fig. 2.4B and C) comparably to whole splenocytes (Fig. 2.1A) and were reduced, when the allogeneic DCs did not express CD40 (Fig.2.4B and C). In contrast, the DC-depleted fraction, which consists mainly of B cells, did not induce proliferation or cytotoxicity independent of CD40 expression (Fig. 2.4B and C).

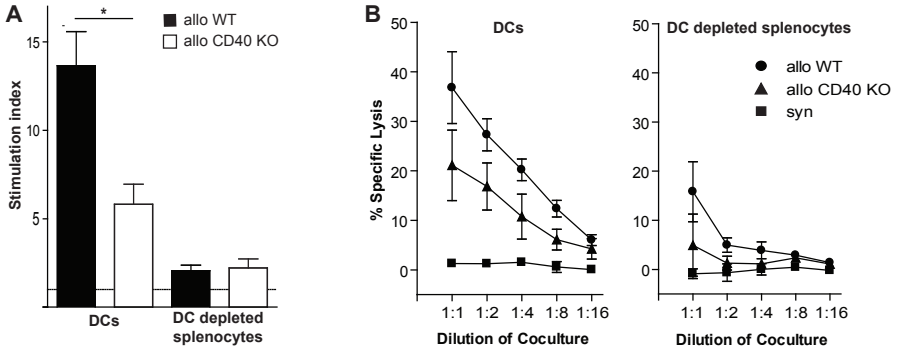

Figure 2.4: CD40 on allogeneic DCs is critical for cytotoxic T cell responses *in vitro*. CBA CD4 and CD8 T cells were cultured in a ratio of 2:1. Splenic DCs from B6 WT or CD40 KO animals were isolated using MACS specific for CD11c. The positive and negative fraction were irradiated and used as stimulators. (A) Proliferation was measured on d 4 of culture. $^*P < 0.05$ (B) Allospecific cytotoxicity was measured on d 5 in a Cr-release assay against IFN-stimulated rTECs from B6 WT mice.

The direct CD8 alloresponse depends on donor CD40 *in vivo*

To evaluate the role of donor CD40 on direct alloreactive CD8 T cells *in vivo*, we performed skin grafts from WT B6 or CD40 KO mice to bm1 mice. In this strain combination K^b is the only mismatched MHC molecule recognized by directly alloreactive CD8 T cells. A prolongation of graft survival by 6 days for CD40 KO grafts compared to WT grafts was observed in this model (Fig. 2.5A). This result suggests that donor CD40 is required for full activation of direct alloreactive CD8 T cells.

To test this experiment in a complete MHC-mismatched situation, we used CBA mice as recipients for WT B6 or CD40 KO skin grafts (Fig. 2.5B). In this case, no difference in allograft survival could be detected. Thus, blocking only donor CD40 is not sufficient to prevent allograft rejection by a graft with MHC class I and II disparity.

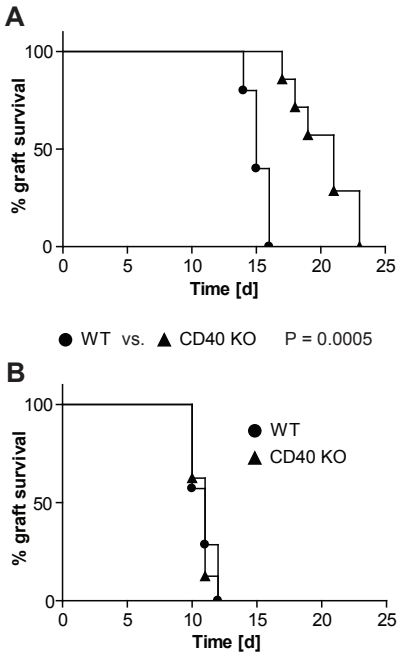

Figure 2.5: MHC class I-mismatched skin graft survival is prolonged in the absence of donor CD40. (A) Skin from B6 WT or CD40 KO donors was transplanted on MHC class I-mismatched bm1 recipients ($n = 5$ for WT grafts, $n = 7$ for CD40 KO grafts). Median survival: 15 vs. 21 days, $P = 0.0005$. (B) Skin from B6 WT or CD40 KO donors was transplanted on fully MHC-mismatched CBA recipients ($n = 7$ for WT grafts, $n = 8$ for CD40 KO grafts). Median survival times: WT and CD40 KO: 11 days.

Absence of CD40 on renal grafts leads to improved allograft function

To analyze the effect of donor CD40 on renal allograft rejection *in vivo*, we performed life-supporting kidney grafts from WT B6 and CD40 KO donors to completely MHC-mismatched CBA recipients. Syngeneic transplants were used as controls. One week post transplantation allogeneic grafts demonstrated severe interstitial infiltrates (Fig. 2.6B and C) and at times prominent tubulitis (Fig. 2.6H). The infiltrates showed a perivenular accentuation (Fig. 2.6G). Infiltrates around arteries were common, but signs of acute vascular rejection (subendothelial infiltrates) were only seen occasionally. CD3 positive T cells as well as B cells were present in the interstitial infiltrates (CD3 staining shown in Fig. 2.6D-E, B220 staining not shown). The severity of these findings was comparable between WT and CD40 KO allografts, whereas syngeneic grafts did not show any infiltrates (Fig. 2.6A and D). Interestingly, despite any obvious morphological difference in the conventional histology, recipients of a CD40 KO kidney showed lower blood urea levels compared to those with a WT graft indicating a better graft function (Fig. 2.6I). Furthermore, these animals displayed a better general condition, represented by less body weight loss (data not shown).

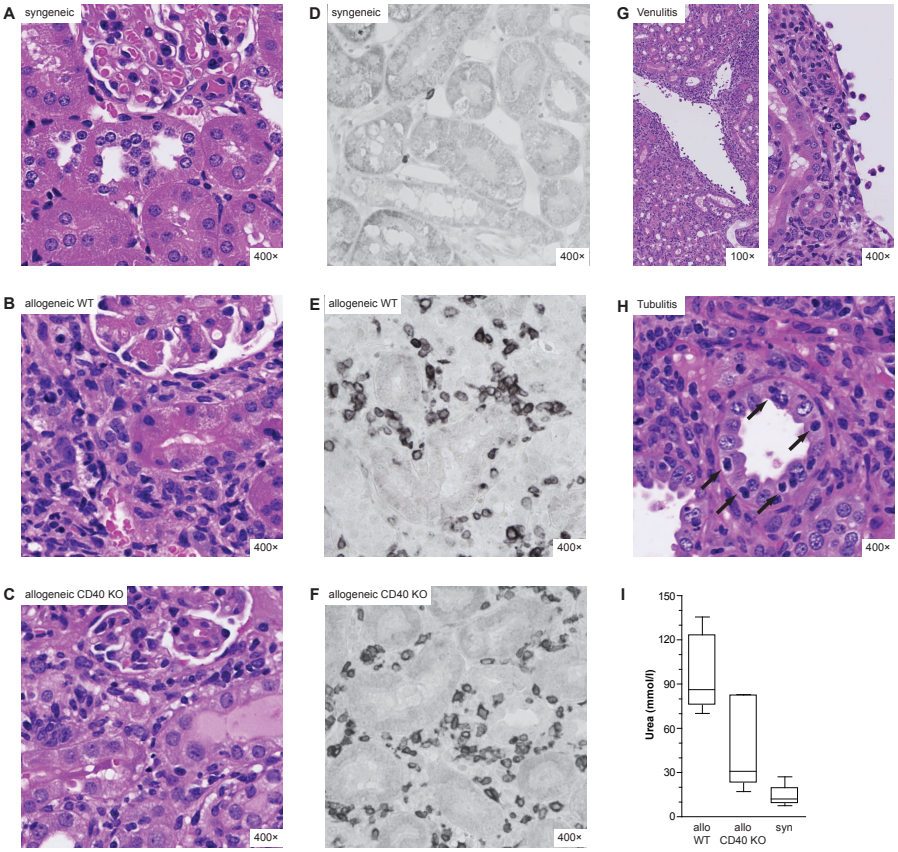

Figure 2.6: Lack of CD40 on renal allografts leads to improved allograft function. B6 WT or CD40 KO mice were used as donors for life-supporting kidney grafts to CBA recipients. Syngeneic controls were performed using B6 WT mice as donor and recipient. Organs were harvested after 1 week ($n = 5$ per group). (A) Representative hematoxylin and eosin stain showing normal renal tissue from a syngeneic graft. (B, C) Representative pictures of hematoxylin and eosin stainings showing interstitial infiltrates in allogeneic grafts. (D-F) Representative pictures of immunohistochemistry for murine CD3. (G-H) Representative pictures of venulitis (G) and tubulitis (H) in allografts. Arrows indicate intraepithelial lymphocytes (H). Magnifications are indicated. (I) Urea concentration in serum of recipients was determined on harvest day.

To further determine the reason for this functional difference, we first examined the intragraft expression levels of inflammatory cytokines such as IFN-γ and IL-17A by qPCR. IFN-γ expression was highly induced in allografts independently of their CD40 expression (Fig. 2.7A). IL-17A was only detectable at very low levels (Fig. 2.7B), and also no difference between WT and CD40 KO grafts was found.

We have previously shown that rTECs, dependent on the surrounding cytokine milieu, do express the costimulatory CD40, but also the coinhibitory PD-L1 (Fig. 2.2) (Starke *et al.* 2007). We therefore tested for PD-L1 expression in the kidney grafts by qPCR. PD-L1 expression was increased in the CD40 KO allografts compared to the WT group (Fig. 2.7C). Furthermore, we detected a

lower amount of apoptotic epithelial cells in this group (Fig. 2.7D and E). Thus, rTECs in a CD40 KO allograft seem to be partially protected from the attack of alloreactive T cells, and this might be mediated by PD-L1, as previously shown in humans (Starke *et al.* 2010).

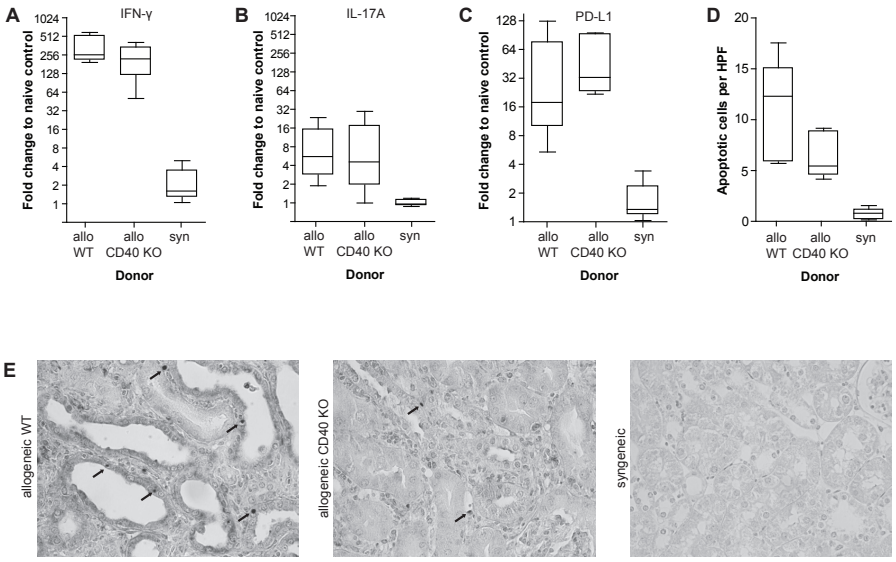

Figure 2.7: Higher expression of PD-L1 and less apoptosis in CD40 KO renal allografts. B6 WT or CD40 KO mice were used as donors for life supporting kidney grafts to CBA recipients. Syngeneic controls were performed using B6 WT mice as donor and recipient. Organs were harvested at 1 week ($n = 5$ per group). (A-C) Intragraft mRNA expression levels of IFN-γ (A), IL-17A (B), and PD-L1 (C) were detected by qPCR. All results are shown as fold change to naive control kidney samples. (D, E) Immunohistochemistry for ssDNA was performed on all grafts. Quantitative analysis (D) and representative pictures (magnification: $\times$400) (E) are displayed. Arrows indicate apoptotic cells. In all experiments groups were compared using a Man-Whitney test. No significant differences between WT and CD40 KO groups could be detected.

Discussion

Directly alloreactive T cells are important effector cells causing acute rejection of solid organ allografts (Benichou *et al.* 1999). In this study we demonstrate a dependence of directly alloreactive T cells on CD40 expressed on donor cells. All effector functions including proliferation, cytokine production, and cytotoxicity were reduced in the absence of CD40 on donor APCs. This was also true for alloreactive CD8 T cells. A connection was found between CD40 signaling and the induction of Th17 cells *in vitro*. *In vivo*, absence of donor CD40 led to prolonged survival of MHC class I-mismatched skin allografts and improved function of fully mismatched renal allografts, potentially mediated by PD-L1-dependent protection of rTECs from apoptosis.

An important role of Th17 cells in response to bacterial pathogens and autoimmune diseases is nowadays widely accepted (reviewed in (Hu *et al.* 2010)).

However, their role in allograft rejection is not completely established yet. The most convincing studies showing, that IL-17 producing T cells can cause allograft rejection, were performed in mice lacking the Th1 specific transcription factor T-bet (Burrell *et al.* 2008; Yuan *et al.* 2008). However, it remains unclear, whether the processes observed in these studies are also taking place in animals sufficient in Th1 responses. Moreover, the impact of IL-17 on the intragraft modulation of the immune response in renal allografts remains to be further investigated.

It has been shown that the induction of virus-specific Th17 cells is dependent on antigen dose and TLR triggering in the APCs, and this process is boosted by triggering CD40 and completely abolished, when CD40 signaling is blocked (Iezzi *et al.* 2009). In our study, we show for the first time that the same is also true for alloreactive T cells. However, we found that the ability of CD4 cells to produce IL-17A is not required to induce full cytotoxic activity in CD8 T cells. This finding is supported by a recent publication of a tumor model, in which Th17 cells help to activate CD8 T cells, but this process was independent of IL-17A and rather mediated by IL-2 and indirect presentation of antigen on MHC class I molecules of the CD4 helpers (Ankathatti Munegowda *et al.* 2010).

When searching for IL-17A expression in acutely rejecting renal allografts, we detected only very low amounts, with no difference between WT and CD40 KO allografts. This might be due to the time point of measurement. Loong *et al.* detected IL-17 producing mononuclear cells in renal allografts as early as day 2 after transplantation (Loong *et al.* 2002). We harvested the renal allografts only at day 7, and this time point might be too late to detect the described effects.

The role of CD40 signaling for T cell activation has been widely studied and in the case of CD4 T cells its role for their activation and cross-talk with B cells is firmly established. However, the role of CD40 signaling to CD8 T cells is less clear. In our model we found that directly alloreactive CD8 T cells not only depended on CD4 help, but also needed a CD40 signal from the donor APCs for complete activation. This result is consistent with an earlier study performed in CD154 KO animals (Zhai *et al.* 2002). However, these animals also lack CD154 on CD4 T cells leading to impaired CD4 help. Here we show for the first time dependency of alloreactive CD8 T cell activity on CD40 signaling *in vitro*, even when CD4 help is intact. Similarly Hernandez *et al.* found a reduced activation of virus-specific CD8 T cells *in vivo*, when CD40-CD154 interaction was disabled specifically between CD8 T cells and DCs (Hernandez *et al.* 2007).

We showed that CD40 expression on donor cells is necessary for cytotoxic CD8 activity during the induction phase. The cells mediating this effect were donor DCs. Tissue residing DCs are the most prominent population of passenger leukocytes. Thus, CD40 blockade early after transplantation might reduce induction of directly alloreactive CD8 T cells by this stimulator population. This is supported by the results obtained in a skin graft model, which is mainly restricted to directly alloreactive CD8 activity. A significant, although rather mild effect on graft survival was observed, which correlated with the *in vitro* results, where cytotoxicity was also significantly reduced, but not abolished,

when the stimulator cells did not express CD40.
Despite reduction of alloreactive T cell responses *in vitro*, severity of kidney allograft rejection assessed by conventional histology was not altered, when the allograft did not express CD40. Also intragraft levels of proinflammatory cytokines (IFN-γ and IL-17A) were identical. Limiting to the model used here is the fact, that one cannot exclude indirect or semi-direct alloantigen presentation on syngeneic APCs with normal CD40 expression. However, we detected improved function of CD40 KO kidney allografts 7 days post-transplantation. rTECs express CD40 under inflammatory conditions. When CD40 is triggered, this leads to activation and subsequent production of inflammatory molecules, such as IL-6, IL-8, RANTES, MCP-1, IL-15 and PAI-1 (Woltman *et al.* 2000). These chemokines and cytokines in turn can cause tubular injury and accelerate renal allograft rejection (Dugger *et al.* 2009). The fact that we found less apoptotic rTECs in CD40 KO grafts, suggests that these cells were partially protected from this process. Similar results were described in a study of chronic proteinuric renal disease, where blockade of CD154 with MR1 led to amelioration of disease (Kairaitis *et al.* 2003). Another reason for less apoptosis found in CD40 KO grafts could be the higher expression of the inhibitory ligand PD-L1. The expression of PD-L1 has been shown to protect human rTECs from allospecific CD8 cytotoxic activity (Starke *et al.* 2010). *In vitro* we showed that IL-17A stimulation in the presence of IFN-γ induces a reduction in PD-L1 expression on rTECs, which is accompanied by an upregulation of CD40 on WT cells. If our *in vitro* results concerning the reduced Th17 induction by CD40 KO donor cells are true also for this *in vivo* model, one would expect a reduced Th17 response in recipients of CD40 KO allografts. The reduced PD-L1 expression in WT allografts might consequently be due to a higher IL-17A concentration early after engraftment concomitant with the high IFN-γ concentration that we found. Thus, the rTECs in WT B6 allografts adopted a more costimulatory then inhibitory state and were more susceptible to T cell mediated cytotoxicity.
We postulate that these two mechanisms - less activation and better protection from CTLs - lead to a better preserved renal function in CD40 KO renal allografts. Whether the surface expression pattern of PD-L1 and CD40 and lower apoptosis level are directly linked in this model needs to be further investigated. Taken together, our results indicate that blocking donor CD40 not only reduced directly alloreactive CD8 T cell responses and Th17 induction, but might additionally prevent rTEC activation and killing resulting in preservation of allograft function. Considering the complications, which blockade of CD154 caused in non-human primates and in humans (Kawai *et al.* 2000), the blockade of CD40 itself may be a more promising strategy to prevent renal allograft rejection.

Section 2
Development of a blocking F(ab)-Fragment against murine CD40

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Contribution to this manuscript:

All experiments were performed by Anna K. Kraus. The manuscript was written and the figures were made by Anna K. Kraus.

Abstract

Blocking the CD40-CD154 costimulation pathway is a promising tool for induction of immunologic tolerance - either to auto- or allo-antigens - or as an alternative to current immunosuppressive treatments. So far most strategies relied on blocking CD154. This, however, has become impossible to use in humans because of thromboembolic events obsereved in the first trials. Thus, CD40 blockade is now in focus of research. Some human or chimeric antibodies against CD40 have already been tested in transplantation models in non-human primates. For mouse, the most used model organism, there are no antibodies against CD40 without activating properties. We therefore developed a F(ab)-fragment binding firmly to murine CD40 without activating CD40 signaling and thereby the cell expressing it. F(ab)86 is able to block CD40 induced signaling, proliferation, and activation in murine B cells. Furthermore, it is a potent inhibitor of allospecific T cell proliferation and Th17 induction *in vitro*.

Introduction

Costimulation blockade is a promising strategy to induce tolerance to various antigens. Especially the blockade of the CD40-CD154 pathway showed good results in several preclinical models for autoimmune diseases and transplantation.
Several autoimmune diseases have been shown to at least partially depend on the CD40-CD154 costimulatory pathway. CD40 and CD154 positive cells have been shown to be present in white matter lesions in multiple sclerosis (MS), and blockade of CD154 was able to ameliorate EAE, a model for MS (Gerritse *et al.* 1996). Also, in models for SLE blockade of CD154 postpones onset of disease in susceptible mice (Mohan *et al.* 1995), and CD28 pathway blockade further enhances this effect (Daikh *et al.* 1997). Finally, in a model for autoimmune arthritis, RNAi-mediated CD40-CD154 interruption led to antigen specific tolerance and prevented disease onset (Zheng *et al.* 2010).
Induction of antigen-specific tolerance is a major goal in transplantation immunology. Despite the improvements of immunosuppressive treatment and reduction of acute rejection episodes during the first year of transplantation in renal transplantation, many grafts are lost in the long term due to rejection or the toxic effects of immunosuppressive drugs (Hariharan *et al.* 2000). The induction of donor-specific tolerance would solve both problems. Several experimental protocols are used in murine and non-human primate models for solid organ transplantation. Most of them contain blockade of the CD40-CD154 pathway. These include simultaneous blockade of the CD40 and CD28 pathways at the time of transplantation (Kirk *et al.* 1997; Larsen *et al.* 1996). Others use DST under CD154 blockade (Markees *et al.* 1997; Phillips *et al.* 2003). A protocol combining anti-CD154, DST and an mTOR-inhibitor has been successfully translated into a non-human primate model for renal transplantation (Preston *et al.* 2005). Finally, the induction of mixed chimerism to achieve stable donor specific tolerance is pursued. For this approach the actual minimal treatment protocols in mice is using CD154 blockade combined with non-myeloablative irradiation and bone marrow transplantation (Fehr *et al.* 2005; Takeuchi *et al.* 2004; Wekerle *et al.* 1999).
Almost all mentioned applications for CD40-CD154 blockade are dependent on antibodies against CD154. However, the use of a blocking CD154 antibody has led to unexpected thromboembolic events in clinical trials (Kawai *et al.* 2000; Knechtle *et al.* 2001), which were due to platelet activation by the administered antibody (Koyama *et al.* 2004). Therefore, efforts were made to find antibodies blocking CD40 and these were successful in non-human primates (Badell *et al.* 2012; t Hart *et al.* 2005). Up to today only antibodies with agonistic activities are available against murine CD40 (e.g. FGK4.5, 3/23, or HM40-3). These multivalent antibodies have the ability to crosslink CD40 and thereby activate it. Furthermore, the administration of antibodies in patients is not problem-free: immunogenicity of not completely humanized antibodies and infusion reactions have been observed with translation to the clinics (Ferran *et al.* 1991; Helliwell and Coles 2009).

In this study we therefore searched to identify a monovalent F(ab)-fragment specific for murine CD40 with antagonisitic properties. In cooperation with AbD Serotec, we found four candidate molecules by phage display library screening. From these four candidates, one molecule was chosen according to its binding properties and activity. This fragment showed a potent ability to block CD40 signaling in B cells and allospecific T cell activation.

Material and Methods

Mice

C57BL/6 (B6, H-2^b), CD40 knock out (CD40 KO, B6 background, H-2^b), and CBA (H-2^k), mice were housed in specific pathogen-free conditions at the University of Zürich. All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office of the Canton of Zürich).

Proliferation assays

T cell proliferation assays were performed using isolated CD4 and CD8 positive T cells as responders. T cells were sorted from whole spleen by magnetic cell separation (MACS) according to the protocols of Miltenyi Biotec (Bergisch Gladbach, Germany). Purity of sorted cells was confirmed by FACS analysis. T cells were then stimulated with irradiated (30 Gy) splenocytes from allogeneic and syngeneic mice.
Proliferation was measured by incorporation of tritium-labeled thymidine (Perkin Elmer, Waltham, USA) on day 4 of culture. Stimulation indices were calculated as follows:

$$\text{Stimulation index} = \frac{\text{incorporation allogeneic sample}}{\text{mean incorporation of syngeneic controls}}$$

B cell proliferation assays were performed using MACS-isolated B cells. B cells were stimulated with a monoclonal anti-IgM antibody (II/41, eBioscience, Frankfurt, Germany) or an anti IgM-F$(ab)_2$-Fragment (polyclonal, Jackson Immunoresearch Europe, Suffolk, UK) coated on the plate at 1 μg/ml in phosphate buffered saline (PBS) over night and a soluble monoclonal anti-CD40 antibody (FGK4.5, Bioxcell, West Lebanon, NH, USA) at 5 μg/ml.

FACS and cytokine quantification

FACS was performed with a BD-FACSCanto II (Becton Dickinson, Allschwil, Switzerland). Anti-mouse CD3-FITC, CD4-PE, CD8-APC, CD45R/B220-PE, CD45/B220-PE-Cy7, CD86-PE, and propidium iodide (PI) were purchased from eBioscience (Frankfurt, Germany), carboxyfluorescein succinimidyl ester (CFSE) from Promega (Dübendorf, Switzerland). A PE-labeled antibody against a 6×His-Tag was purchased from Abcam (Cambridge, UK). Analysis of raw data was performed using FlowJo 7.6.1 software (Tree star Inc., Ashland, OR, USA).

Cytokines from cell culture supernatants were quantified using a FlowCytomix set purchased from eBioscience (Frankfurt, Germany) according to the manufacturer's manual.

Antigen production and phage display library screen

The murine plastocytoma cell line expressing mCD40-huγ1, a fusion protein consisting of murine CD40 linked to a human IgG heavy chain, was kindly provided by David Gray (Gray *et al.* 1994). The fusion protein was purified from cell culture supernatants using a gravity flow protein A column according to manufacturer's instructions (Thermo Fisher Scientific Inc., Rockford, IL, USA). The resulting protein solution was rebuffered to PBS. Protein concentration was measured using a BCA assay (Thermo Fisher Scientific Inc., Rockford, IL, USA). Purity of the fusion protein was detected by SDS polyacrylamide gel electrophoresis.
The mCD40-huγ1 protein together with the negative control (human IgG heavy chain) served as target for a screen of the HuCAL ® PLATINUM phage display library (Knappik *et al.* 2000, Prassler *et al.* 2011) performed by AbD Serotec (Puchheim, Germany). Binding of the resulting F(ab)-fragments to the target was proven by ELISA performed by AbD Serotec (Puchheim, Germany).

Chemicals

Phorbol$_1$2-myristate$_1$3-acetate (PMA) and ionomycin were purchased from Sigma Aldrich (Buchs, Switzerland) and used at 50 ng/ml and 500 ng/ml, respectively.

Western blotting

Western blot analysis was performed using mouse anti IκB alpha antibody (Abcam, Cambridge, UK) and a mouse anti β-tubulin antibody (Sigma Aldrich, Buchs, Switzerland). A peroxidase-conjugated goat anti-mouse IgG was used as secondary reagent. Determination of band density was performed using ImageJ software (Wayne Rasband, National Institute of Health, USA).

Statistical analysis

All statistical comparisons were performed with GraphPad Prism 4. Normally distributed groups were compared using Student's t test. Groups without Gaussian distribution were compared using the Mann-Whitney test. $P < 0.05$ was considered significant.

Results

Generation of blocking F(ab)-fragments against murine CD40

As target protein for a phage display library screen of the HuCAL ® PLATINUM library performed by AbD Serotec we used a fusion protein consisting of murine CD40 and a human IgG heavy chain (mCD40-huγ1). MCD40-huγ1 was purified from cell culture supernatant of the stably transfected plastocytoma cell line using a protein A column, which binds specifically the human IgG heavy chain in the fusion protein. After rebuffering to PBS, purity of the gained protein was analyzed by SDS polyacrylamide gel electrophoresis (Fig. 2.8A). This protein together with a negative control (human IgG heavy chain) was sent to AbD Serotec as target for the phage display library screen. Four candidate F(ab)-fragments were obtained from this. Their binding to mCD40-huγ1 was proved by ELISA performed by AbD Serotec (Fig. 2.8B). F(ab)-fragment AbD0866786.1 (F(ab)86) bount the target with the highest affinity.

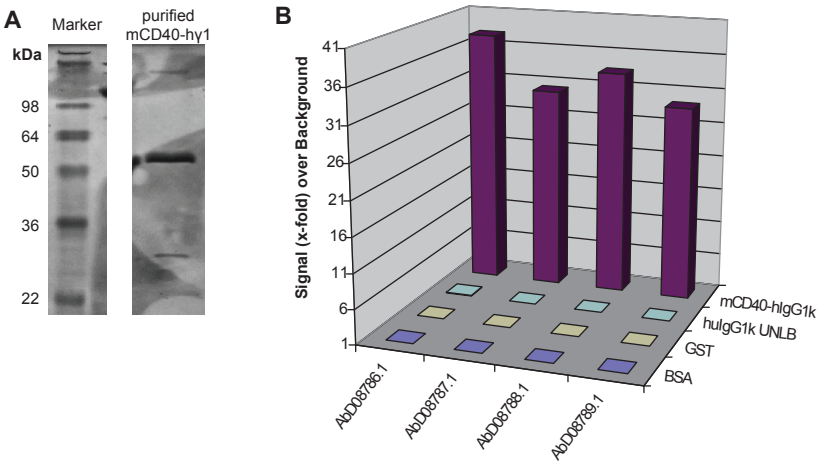

Figure 2.8: Screening for a blocking anti-CD40 F(ab)-fragment. (A) mCD40-huγ1 was purified over a protein A column and rebuffered to PBS. Purity was analyzed by SDS polyacrylamide gel electrophoresis. (B) Binding of the four F(ab)-fragment candidates to mCD40-huγ was assessed by ELISA performed by AbD Serotec. Human IgG heavy chain alone, BSA and GST were used as negative controls.

Screening for the best candidate F(ab)

B cells express CD40. To identify the best of these four candidate fragments, we used murine B cells and analyzed binding and proliferation inhibition as readouts. All fragments were incubated with B6 splenocytes and detected with an antibody against the 6×His-Tag, which all fragments contained. All four fragments bound to B cells (Fig. 2.9A). The strongest binder was F(ab)86 (Fig. 2.9A). This correlated with the results for solid phase binding obtained by ELISA performed by AbD Serotec.

We then performed a functional screen using a B cell proliferation assay based on CFSE dilution. MACS-isolated B cells were labeled with CFSE and stimulated with a plate bound anti-IgM and a soluble anti-CD40 antibody (FGK4.5). After four days the cells were harvested and CFSE dilution was detected by FACS. Combined stimulation of IgM and CD40 induced B cell proliferation. This could be blocked most efficiently by F(ab)86 (Fig. 2.9B), but not with the other candidate fragments. Thus, we chose F(ab)86 to proceed with further experiments.

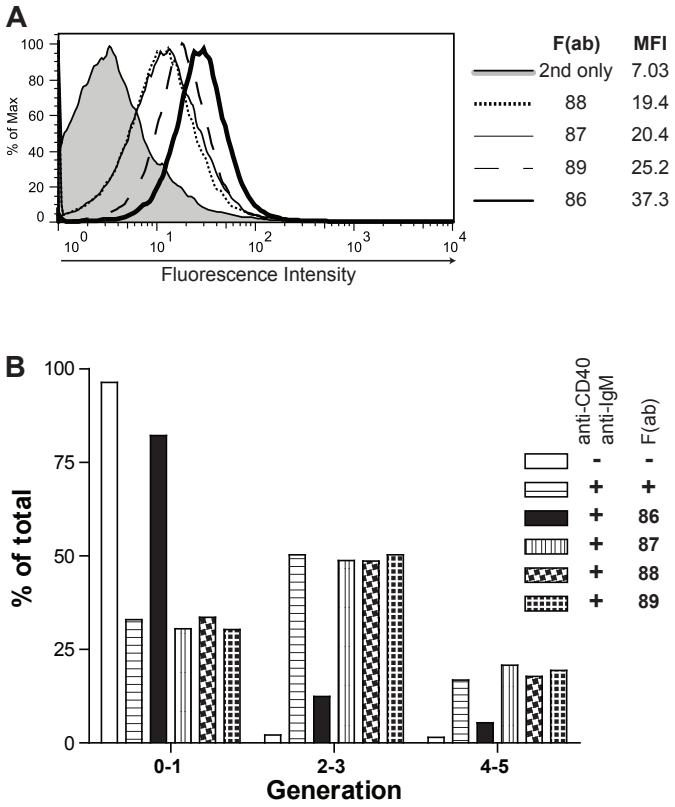

Figure 2.9: Screening for a blocking anti-CD40 F(ab)-fragment. (A) Splenocytes from B6 mice were incubated with equal amounts of all four candidate F(ab) fragments. Binding of F(ab)-fragments to B220 prositive B cells was detected with a secondary antibody directed against the 6×His-Tag and analyzed by FACS. Mean fluorescence intensities (MFIs) are indicated. (B) Isolated splenic B cells from B6 mice were labeled with CFSE and stimulated with plate bound anti-IgM antibody (1 μg/ml) and soluble FGK4.5 (5 μg/ml) or left untreated for 4 days. F(ab)-fragment candidates were added from the beginning of culture at 5 μg/ml. Proliferation was assessed by CFSE dilution in FACS.

F(ab)86 reduces CD40 driven B cell activation and proliferation

We used the more sensitive method of ^{3}H-thymidine-incorporation to measure B cell proliferation. Isolated B cells were stimulated with plate-bound anti-IgM and soluble FGK4.5. Proliferation was measured on day 4. Titrated amounts of Fab(86) were added to the culture in order to block B cell activation and proliferation. At a concentration of 5 μg/ml F(ab)86 reduced B cell proliferation to negative control level (Fig. 2.10A).
To assess B cell activation we assessed the expression of the activation marker CD86 on day 4 of stimulating culture. F(ab)86 strongly reduced upregulation of CD86 induced by CD40 stimulation from a concentration of 5 μg/ml (Fig. 2.10B). The median fluorescence intensity was reduced from 101 for the stimulated control to 18 at 5 μg/ml or 16.3 at 10 μg/ml (median fluorescence intensity untreated control: 9.46).

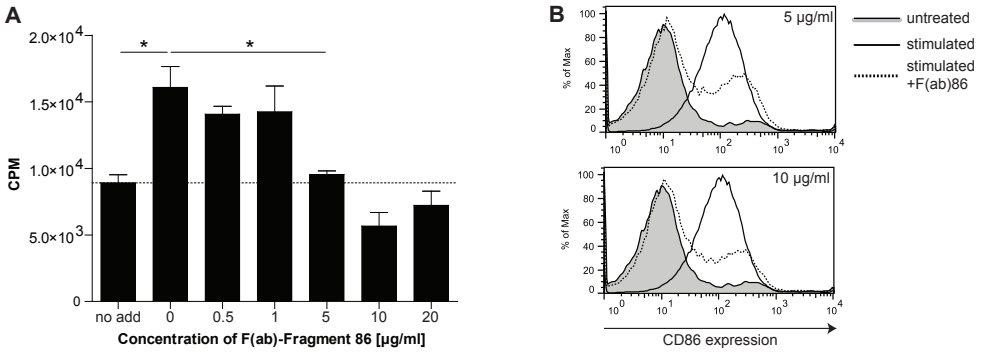

Figure 2.10: F(ab)86 reduces CD40 driven activation and proliferation. Isolated splenic B cells from B6 mice were stimulated with plate bound anti-IgM F(ab)$_2$-fragment (1 μg/ml) and soluble FGK4.5 (5 μg/ml) or left untreated for 4 days. Titrated amounts of F(ab)86 was added to stimulating cultures at indicated concentrations. (A) Proliferation was measured by 3H-Thymidine incorporation. * $P < 0.015$. (B) Expression of the activation marker CD86 was assessed by FACS.

F(ab)86 blocks CD40-induced NFκB signaling

Trimerization-induced recruitment of tumor necrosis factor receptor associated factors (TRAFs) 2, 3, 5, and/or 6 to the cytoplasmic domain of CD40 triggers the canonical NFκB pathway, which induces the ubiquitin- and proteasome-dependent degradation of IκB and the subsequent release of NFκB, which then translocates to the nucleus (Elgueta *et al.* 2009). We therefore tested, if F(ab)86 is able to block the degradation of IκB. Isolated splenic B cells were incubated on plate-bound FGK4.5 for 2 hours. F(ab)86 was added at indicated concentrations from the beginning. Protein lysates were prepared and used for a western blot specific for IκB. As positive control we used B cells stimulated with PMA and ionomycin. IκB degradation in B cells was induced after 2 hours of incubation with FGK4.5 (Fig. 2.11A and B). This was reversed and IκB content was restored already at a concentration of 5 μg/ml F(ab)86 (Fig. 2.11A and B).

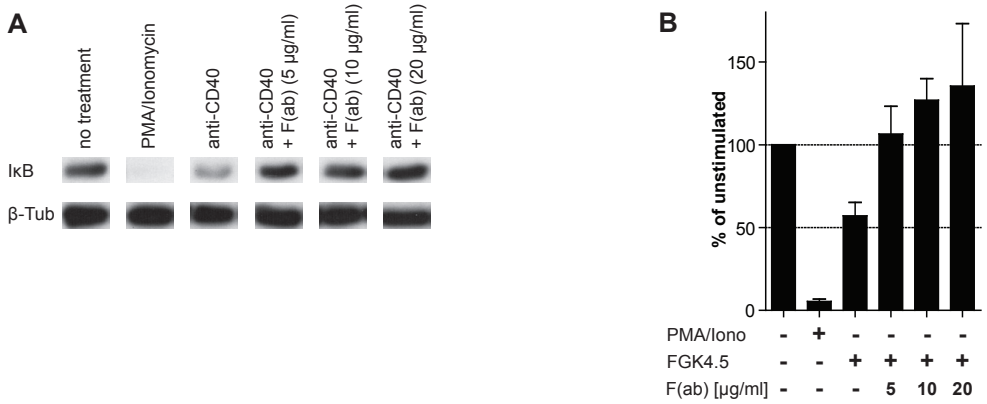

Figure 2.11: F(ab)86 blocks NFκB signaling. Isolated splenic B cells from B6 mice were incubated on plate bound FGK4.5 (5 μg/ml) for 2 hours and F(ab)86 was added at indicated concentrations. IκB content of the cytoplasm was detected by western blot. As loading control staining for β-tubulin was used. (A) Representative western blot for IκB. (B) Density measurement was performed using the ImageJ software. Density of IκB bands was normalized to that of β-tubulin bands, and IκB content of untreated B cells was set as 100%. Relative content of IκB in treated B cells is shown.

F(ab)86 blocks allospecific T cell activation and Th17 induction

The CD40-CD154 pathway is also important for the priming of T cells. Not only does CD154 expressed on the T cell give an activation signal to the T cell (van Essen *et al.* 1995), but also the engagement of CD40 leads to activation and further upregulation of costimulatory molecules on the APC (Quezada *et al.* 2004). We therefore tested, if blocking CD40 with F(ab)86 can reduce allospecific T cell proliferation *in vitro*. CBA T cells (H-2^k) were isolated and stimulated with completely MHC-mismatched splenocytes from B6 mice (H-2^b). As controls we used stimulators deficient in CD40 (H-2^b) and syngeneic splenocytes. The addition of F(ab)86 reduced allospecific T cell proliferation in a dose-dependent manner. At 10 μg/ml T cell proliferation was similar as when using CD40 KO stimulators. We have previously shown that in a similar *in vitro* assay the induction of Th1 cells is partially and the one of Th17 cells critically dependent on CD40 expression on the stimulator cells (see section 2). We thus measured cytokine content in the supernatants of these cocultures. For IFN-γ a reduction to the level achieved with CD40 KO stimulators was reached already at 1 μg/ml F(ab)86 (Fig. 2.12B). Addition of the higher concentration further reduced IFN-γ to a non detectable amount (Fig. 2.12B). The induction of Th17 cells was monitored by the production of IL-6 and IL-17A. Complete blockade of Th17 development needed a higher concentration of F(ab)86 and was achieved at 10 μg/ml (Fig. 2.12B).

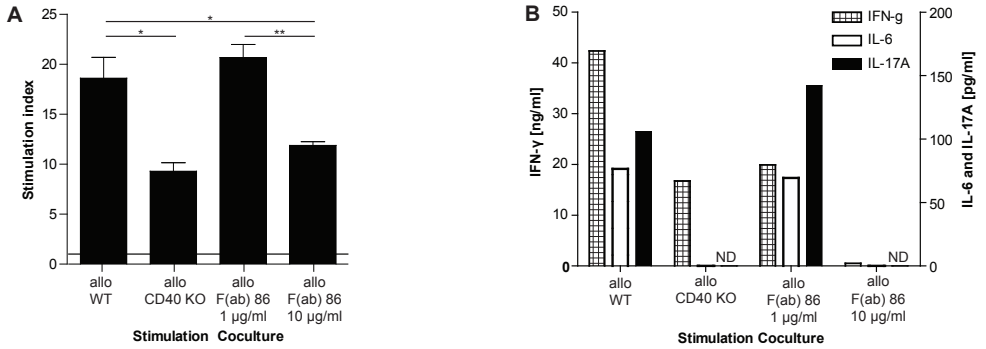

Figure 2.12: F(ab)86 reduces allospecific T cell proliferation. Isolated splenic CD4 and CD8 T cells from CBA mice (H-2^k) (ratio 2:1) were stimulated with irradiated (30 Gy) splenocytes from B6 mice (H-2^b). F(ab) 86 was added at indicated concentrations from the beginning of culture. As controls stimulators from allogeneic CD40 KO (H-2^b) and syngeneic cells were used. (A) Proliferation was measured by ^{3}H-thymidine incorporation on day 4. Stimulation indices relative to syngeneic control are shown. $^*P < 0.04$, $^{**}P < 0.004$. (B) Supernatants of cocultures were analyzed for cytokine content on day 4 using a multiplex bead assay. Concentrations for IFN-γ (left axis), IL-17A, and IL-6 (right axis) are shown. ND=not detectable

Discussion

In this study we identified an F(ab) fragment directed against murine CD40 with blocking properties. F(ab)86 was able to block B cell activation and proliferation. We showed that this is due to blockade of CD40 downstream signaling. Furthermore, F(ab)86 blocked directly allospecific T cell proliferation and cytokine production in the same way as the use of CD40 KO stimulators does. The blockade of CD154 appeared as a very promising tool to induce allospecific tolerance or to ameliorate several autoimmune diseases (Fehr *et al.* 2005; Gerritse *et al.* 1996; Kirk *et al.* 1997; Larsen *et al.* 1996; Markees *et al.* 1997; Mohan *et al.* 1995; Phillips *et al.* 2003; Preston *et al.* 2005; Takeuchi *et al.* 2004; Wekerle *et al.* 1999). Unfortunately, the translation of CD154 blockade into clinics in studies with renal transplant recipients led to severe thromboembolic events (Knechtle *et al.* 2001), which was concomitantly observed in non-human primates (Kawai *et al.* 2000), and was eventually stopped. These events have been found to be due to the activation of CD154 on platelets (Koyama *et al.* 2004).

As all previous attempts to block the CD40-CD154 pathway relied on antibodies binding to CD154, it was questionable if blocking CD40 is just as effective as blocking CD154. Various antibodies directed against CD40 have already been tested in nonhuman primates. Chi220, a chimeric antibody directed against human CD40 allowed extended islet allograft survival in synergy with CTLA4-Ig (Adams *et al.* 2005). However, this antibody is B cell-depleting (Adams *et al.* 2005). Recently, another anti-CD40 antibody 3A8 was successfully tested in islet transplantation and induction of mixed chimerism in rhesus macaques (Badell *et al.* 2012; Page *et al.* 2012). 3A8 is a mouse antibody directed against

human CD40, which is not B cell-depleting. 3A8 was able to suppress allospecific T cell proliferation. However, it was not able to block binding of soluble CD154 to CD40, and it induced upregulation of B7 molecules on B cells *in vitro* (Badell *et al.* 2011). Still, the results from studies using these antibodies indicate that blockade of the CD40/CD154 pathway on the receptor side is as beneficial as blocking the ligand CD154.
In fact, we showed that F(ab)86 is a potent inhibitor of B and T cell activation *in vitro* due to its ability to bind CD40 without activating it. Th17 cells have been shown to play a role in the pathogenesis of several autoimmune diseases. Furthermore, Iezzi *et al.* showed that the CD40-CD154 pathway is crucial for the induction of Th17 cells in a murine EAE model (Iezzi *et al.* 2009). F(ab)86 is not only capable of inhibiting T cell proliferation. We also detected a strong inhibition of Th17 induction. Blocking induction of this T cell subset might therefore be another promising application of F(ab)86.
Monoclonal antibodies found their way into clinics for many different diseases (Getts *et al.* 2010). However, even if these antibodies are humanized, some of them are still immunogenic and induce anti-drug antibodies. These can reduce treatment efficacy or lead to acute infusion reactions (anaphylaxis due to anti-drug antibodies) (Maggi *et al.* 2011). Some therapeutic antibodies also result in a strong cytokine release upon infusion (Maggi *et al.* 2011). Finally, partially agonistic polyvalent antibodies against costimulatory molecules bear the risk of inflammatory reactions, as shown for an anti-CD28 antibody (Suntharalingm *et al.* 2006). We therefore chose to use an F(ab)-fragment, which avoids receptor mutlimerization and is less immunogenic. However, one significant limitation of F(ab)-fragments is their short half-life *in vivo*. This can be prolonged by modification of the compound. One option for this is pegylation. In this case the polyethylene glycol chains of variable size and grade of branching are bound covalently to the F(ab)-fragment. Thereby size and hydrophily are increased and immunogenicity decreased (Veronese 2002). Similary modified cytokines, for example IFN-α for the treatment of hepatitis B and C, are already in clinics for several years (Papatheodoridis and Cholongitas 2004). However, pegylation has also been successfully used to increase the half life of an $F(ab)_2$-fragment directed against IL-8 (Koumenis *et al.* 2000) and various F(ab)-fragments in rodent studies (Baer *et al.* 2009; Chapman *et al.* 1999). Finally, a pegylated F(ab)-fragment directed against human TNF-α made its way to the clinics for treatment of rheumatoid arthritis and Crohn's disease (Choy *et al.* 2002; Sandborn *et al.* 2007). Another option for an increase in half life would be the generation of a fusion protein containing F(ab)86 and a bigger but rather inert fusion partner like albumin (Smith *et al.* 2001) or α1-antitrypsin (Vanhove *et al.* 2003). The latter has been pursued for an Fv-single chain directed against human CD28. Functionality of the Fv-fragment was given and half life could be extended to a useful amount of time (Vanhove *et al.* 2003).
Taken together, F(ab)86 is a potent tool to study CD40 blockade in murine models for tolerance induction and autoimmune diseases. Because the screening and selection are quickly to do using the phage display library we used, translation of this approach into larger animals and humans seems feasible.

Section 3
DNAM-1 in renal allograft rejection: Important for T cell priming, but not for cytotoxicity against renal tubular epithelial cells

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All experiments, except renal allografts, histology, and immunohistochemistry were performed by Anna K. Kraus. The manuscript was written and the figures were made by Anna K. Kraus.

Abstract

A new costimulatory pathway is emerging in the field of transplantation research. DNAM-1 and its two ligands have been shown to mediate NK and T cell killing of tumor cells and non professional antigen presenting cells. Blockade of DNAM-1 was beneficial in a model for graft-versus-host disease. Therefore, we tested the importance of DNAM-1 and its two ligands (CD155 and CD112) in renal allograft rejection. We show that both ligands for DNAM-1 are expressed on renal tubular epithelial cells and upregulated in acutely rejected renal allografts and under inflammatory conditions *in vitro*. *In vitro* we detected a role for DNAM-1 in allospecific T cell priming, which however was independent from the ligation of CD155 or CD112. Furthermore, allospecific cytotoxicity against rTECs was independent of DNAM-1 ligation by the targets. In *in vivo* models for solid organ rejection, there was also no effect on severity of allograft rejection, when DNAM-1 ligands were missing. However, in renal allografts deficient in CD155 or CD112, we detected a higher incidence of infarcts indicating endothelial damage. Thus, the blockade of DNAM-1 itself, but not of its ligands might be beneficial in solid organ transplantation.

Introduction

Kidney transplantation is the best therapy option for end stage renal disease (ESRD) (Wolfe *et al.* 1999). Despite improvement of first year graft survival, a lot of grafts are lost in the long term due to rejection and the toxicity of classical immunosuppressant drugs (Hariharan *et al.* 2000). Therefore there is a need for better understanding of the processes that lead to rejection and for new strategies to achieve more specific and less toxic immunosuppression.
For complete activation T cells do not only need the signal via the T cell receptor but also costimulatory signals which can be delivered through several receptors. Costimulation blockade has therefore become a promising tool for immunosuppression and/or tolerance induction in solid organ transplantation. Latest developments focussed mainly on the classical costimulatory molecules B7 and CD40 (Pilat *et al.* 2011).
However, for cytotoxic activity of CD8 positive CTLs intercellular adhesion molecules like LFA-1, LFA-3, and CD2 have been found to play an important role (Shaw *et al.* 1986). Additionally, DNAX accessory molecule-1 (DNAM-1 or CD226) has first been described in the 1990s (Shibuya *et al.* 1996). It is an adhesion molecule of the Ig-family, which in mice is expressed on all CD8 T cells, on activated CD4 T cells and a part of the NK cells (Dardalhon *et al.* 2005). DNAM-1 ligation is crucial for cytotoxic activity of NK and T cells against non-classical APCs like tumor cells (Gilfillan *et al.* 2008; Seth *et al.* 2009; Tahara-Hanaoka *et al.* 2006). Also during priming of CD8 T cells by non-professional APCs like B cells DNAM-1 plays an important role (Gilfillan *et al.* 2008).
DNAM-1 has two known ligands CD155 (Necl-5, PVR) and CD112 (nectin-2) (Fig. 1.5). Both are expressed on a variety of epithelial, endothelial, and antigen presenting cells (Bottino *et al.* 2003; Gilfillan *et al.* 2008; Pende *et al.* 2006; Takai *et al.* 2008). CD155 has a higher affinity to DNAM-1 then CD112 (Reymond *et al.* 2004; Seth *et al.* 2009).
Renal tubular epithelial cells (rTECs) are the major target of allospecific CTLs during cellular renal allograft rejection. They have been shown to act as non-professional APCs under inflammatory conditions, as they express MHC I and II and also the costimulatory molecule CD40 but not CD80 and CD86 (Starke *et al.* 2007; Wuthrich *et al.* 1990).
In this study we therefore investigated the role of DNAM-1 and both of its ligands for allospecific T cell priming and cytotoxicity against renal tubular epithelial cells *in vitro* and *in vivo*.

Material and Methods

Mice

C57BL/6 (B6, H-2^b), CBA (H-2^k), and Balb/c (H-2^d), DBA/2 (H-2^d), and B6.C-H2-K^{bm1}/By (bm1, H-2^{bm1}) mice were housed in specific pathogen-free conditions at the University of Zürich. Bm1 mice express the same H-2 haplotype as B6 (H-2^b) except for 7 nucleotide differences in the gene for H-$2K^b$

resulting in amino acid substitutions at codons 152 (glutamate to alanine), 155 (arginine to tyrosine) and 156 (leucine to tyrosine) (Schulze *et al.* 1983). CD155 KO mice (H-2^d) and CD112 KO mice (H-2^b) were kindly provided by Dr. Günter Bernhardt (Hannover Medical School). All animal experiments were performed according to protocols approved by the legal authority (Veterinary Office of the Canton of Zürich).

Culture of renal tubular epithelial cells

Preparation and primary culture of renal tubular epithelial cells was performed as described (Wuthrich *et al.* 1990). Cells were cultured on collagen-coated dishes in K1 media. In all cytotoxicity experiments primary renal tubular epithelial cells were prestimulated for 48 hours with murine IFN-β and -γ at 100 U/ml each (both purchased from Antigenix America Inc., Huntington Station, NY, USA), prior to use.

T cell proliferation and cell-mediated lympholysis (CML) assay

T cell proliferation and CML assays were performed using either whole spleen or isolated CD4 and CD8 positive T cells as responders. T cells were sorted from whole spleen by magnetic cell separation (MACS) according to the protocols of Miltenyi Biotec (Bergisch Gladbach, Germany). Purity of sorted cells was confirmed by FACS analysis. T cells were stimulated with irradiated (30 Gy) splenocytes from allogeneic and syngeneic mice.
T cell proliferation was measured by incorporation of ^{3}H-labeled thymidine (Perkin Elmer, Waltham, USA) on day 4 of culture.
CML assays were performed on day 5 of culture: ^{51}Chromium (Cr)-labeled, IFN-stimulated allogeneic renal tubular epithelial cells were added to the serially diluted culture for 4 hours (killing phase), and allospecific cytotoxicity was assessed by measurement of Cr-release in the supernatant. Allospecific lysis was calculated as:

$$\% \text{ specific lysis} = \frac{(\text{experimental release} - \text{spontaneous release})}{(\text{total release} - \text{spontaneous release})} * 100$$

In some assays blocking antibodies against mouse DNAM-1 (3B3, Seth *et al.* 2009) or CD112 (6B3, Aoki *et al.*) were added to the culture. These and the matching unspecific isotype control were kindly provided by Dr. Günter Bernhardt (Hannover Medical School). Concentrations are indicated.

FACS

FACS was performed with a BD-FACSCanto II (Becton Dickinson, Allschwil, Switzerland). Anti-mouse CD3-FITC, CD4-PE, CD8-APC, anti rat-IgG-FITC, and propidium iodide (PI) were purchased from eBioscience (Frankfurt, Germany). Rat anti-mouse CD155 was purchased from Biolegend (Fell, Germany) and rat anti-mouse CD112 from Santa Cruz Biotechnology (Heidelberg, Germany).

mRNA isolation and qPCR

mRNA was isolated from kidney grafts or naive kidneys, which had been stored in RNase-inhibitor, using the RNeasy Mini Kit (Qiagen) according to manufacturer's instructions. 1 μg mRNA was transcribed to cDNA using the Omniscript reverse transcription Kit (Qiagen) according to manufacturer's instructions. Pre-developed TaqMan reagents were used for quantitative PCR (Applied Biosystems) detecting murine CD155 and CD112, and the reference 18s rRNA. The expression of candidate genes was normalized to the reference, and fold changes were calculated in relation to the matching controls using the 2^{-ddCt} method.

Skin and kidney grafting

At day 0 full thickness tail skin (about 0.5-1.0 cm^2) from donor mice was transplanted to the dorsal flank area of recipient mice. Graft rejection was defined as graft necrosis $> 90\%$ of the graft.
Kidney grafts were performed in a non-life-supporting manner as previously described in detail (Tian *et al.* 2010). The recipient was unilaterally nephrectomized and orthotopic transplantation was performed on the right side. Allografts were harvested on day 21.

Histology and immunohistochemistry

Histologic examination of all kidney grafts was performed by an experienced renal pathologist blinded to the experimental procedures. Tissues were immersion-fixed in 4% phosphate buffered formalin and embedded in paraffin. The thickness of sections was 4 μm. The slides were routinely stained with hematoxylin and eosin (H & E).
For detection of apoptotic cells by immunohistochemistry the monoclonal antibody F7-26 (Chemicon, International, Inc. Temecula, CA) was used as previously described (Segerer *et al.* 2002). F7-26 binds to single-stranded DNA after thermal denaturation. A peroxidase-conjugated monoclonal rat anti-mouse IgM antibody (Zymed, San Francisco, CA) was used as secondary reagent. Dense, apoptotic nuclei positive for single stranded DNA were quantified in mouse renal allografts in 15 high power fields (original magnification $\times 250$).

Statistical analysis

All statistical comparisons were performed with GraphPad Prism 4. Normally distributed groups were compared using Student's t test. Groups without Gaussian distribution were compared using the Mann-Whitney test. $P < 0.05$ was considered significant.

Results

Ligands for DNAM-1 are expressed in the kidney and upregulated in acute allograft rejection

Expression of both ligands for DNAM-1, CD155 and CD112, has been shown in naive murine kidneys (Nabekura *et al.* 2010). We tested the regulation of expression of both molecules in acutely rejected renal allografts. Quantitative PCR analysis showed an induction of both DNAM-1 ligands compared to naive kidneys (Fig. 2.13A). This induction was not present in syngeneic control allografts. The upregulation of CD155 and CD112 correlated with each other (Fig. 2.13B).
RT-PCR of whole organs does not reveal the exact location of expression. To test whether CD155 and CD112 are expressed on renal epithelium, we stained for them on primary rTECs. FACS analysis showed a constitutive expression of both molecules on WT B6 rTECs (Fig. 2.13B). The addition of proinflammatory cytokines like IFN-β and -γ further increased this expression (Fig. 2.13B). Immunohistochemical staining for CD155 in naive and acutely rejected kidneys is ongoing.

DNAM-1 blockade *in vitro* reduces allospecific T cell activation independently of both ligands

To test for the role of DNAM-1 in allospecific T cell priming, we performed classical *in vitro* MLR assays using isolated T cells from CBA mice stimulated with syngeneic or fully MHC mismatched B6 splenocytes. Either isotype-control or anti-DNAM-1 antibody was added to the coculture from day 0. Proliferation was measured by ^{3}H-thymidine incorporation at day 4. Allospecific proliferation was significantly reduced in all three combinations using CD8 or CD4 T cells alone or both subtypes in combination (Fig. 2.14A). Thus, DNAM-1 signaling is important for both allospecific CD4 and CD8 T cell proliferation.
To identify which ligand of DNAM-1 is important for this effect, we performed MLR assays using allogeneic stimulators deficient in either CD155 or CD112. Both T cell subtypes alone or in combination were stimulated with completely MHC mismatched splenocytes. In none of the cocultures we detected reduction of allospecific proliferation, when stimulators did not express CD155 or CD112 (Fig. 2.14B and C). And contrary to the previously published results, a lack of CD155 on stimulators did increase allospecific proliferation significantly, when CD8 and CD4 T cells were cultured together (Fig. 2.14B). The same was true for CD112: CD4 T cells responding alone and CD4 and CD8 T cells cultured together proliferate significantly more if stimulators do not express CD112 (Fig. 2.14C).

To further test the influence of DNAM-1 on allospecific T cell activation, we tested the ability of similarly stimulated T cells to kill rTECs from allogeneic WT donors. Along with the results for proliferation, the cytotoxic activity against rTECs of T cells stimulated in the presence of a blocking DNAM-1 antibody was reduced (Fig. 2.14D). To test the influence of CD155 and CD112 on T cell priming in this setup, we used T cells stimulated in the presence or absence of CD155 or CD112 on stimulators. In parallel to the results for proliferation, cytotoxicity was not significantly altered and by trend higher, when stimulators did not express CD155 or CD112 (Fig. 2.14E and F).

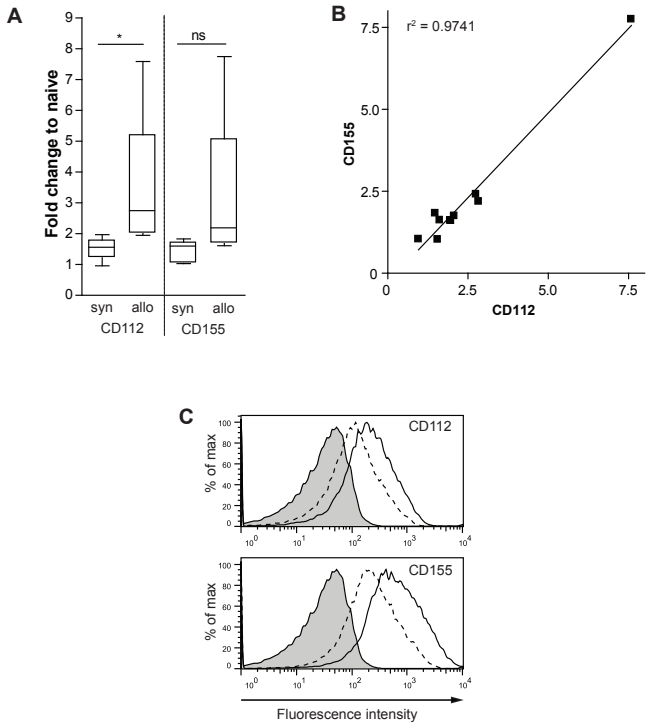

Figure 2.13: Ligands for DNAM-1 are upregulated in acutely rejected renal allografts. (A, B) Renal allografts from B6 to fully MHC-mismatched CBA recipients were performed. Allografts were acutely rejected at day 7 post transplantation. As control syngeneic grafts were used. ($n = 5$ per group) Real time-PCR for CD155 and CD112 was performed on naive kidneys and kidney grafts. (A) The fold upregulation of CD112 and CD155 compared to naive renal tissue is depicted. Groups were compared using the Mann-Whitney-test: $^*P = 0.0159$, ns = not significant. (B) CD112 and CD155 expression are highly correlated. (C) Primary rTECs were left untreated or stimulated with IFN-β and -γ (100 U/ml each) for 48 hours. Surface expression of CD155 and CD112 was analyzed by FACS. Shaded: isotype control, dotted line: unstimulated, solid line: stimulated.

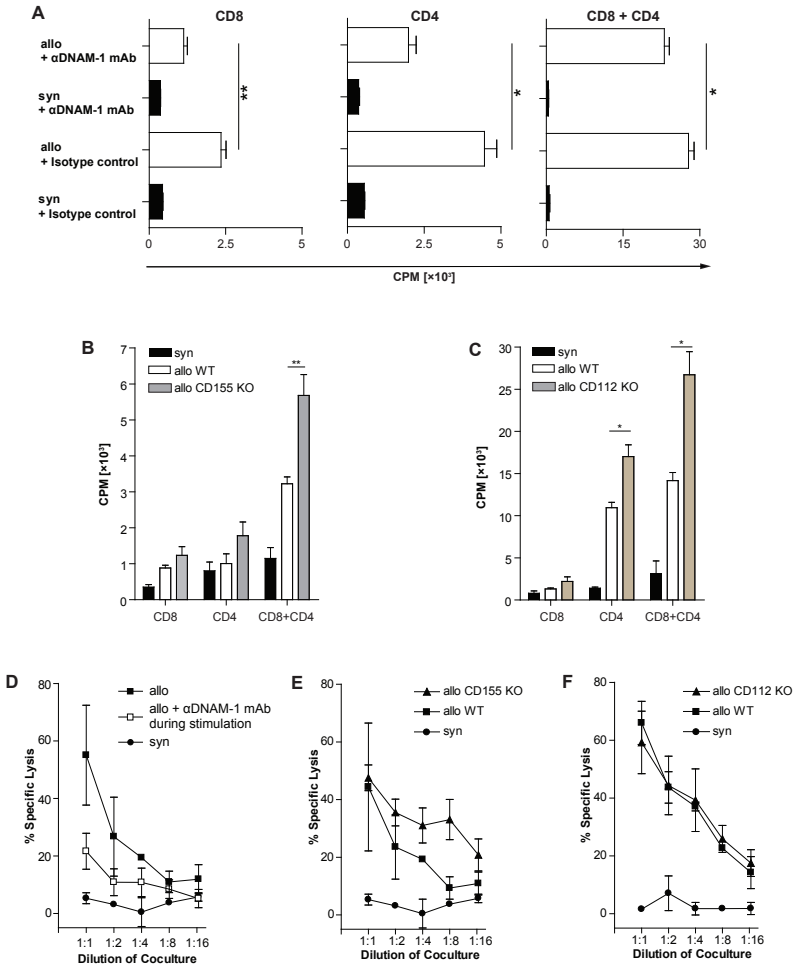

Figure 2.14: DNAM-1 assisted T cell activation is independent of CD155 and CD112 *in vitro*. (A) Isolated CBA T cells were stimulated with irradiated B6 splenocytes. CD8 or CD4 T cells were either cultured alone or in combination (ratio 1:2). To the coculture an agonistic anti-DNAM-1 antibody was added at 25 μg/ml. As control an unspecific isotype control antibody was used at the same concentration. Proliferation was measured on d 4 of coculture. $^*P < 0.035$, $^{**}P < 0.01$. (B) Isolated B6 T cells were stimulated with irradiated Balb/c WT or CD155 KO splenocytes. CD8 or CD4 T cells were either cultured alone or in combination (ratio 1:2). Proliferation was measured on d 4 of coculture. $^{**}P = 0.0025$. (C) Isolated CBA T cells were stimulated with irradiated B6 WT or CD112 KO splenocytes. CD8 or CD4 T cells were either cultured alone or in combination (ratio 1:2). Proliferation was measured on d 4 of coculture. $^*P < 0.018$ (D) CBA splenocytes were stimulated with irradiated B6 splenocytes in presence or absence of anti-DNAM-1 antibody (25 μg/ml). Cytotoxic activity against IFN-stimulated WT B6 rTECs was measured on d 5. (E) Isolated CD8 and CD4 T cells (ratio 1:2) were stimulated with irradiated WT Balb/c or CD155 KO splenocytes. Cytotoxicity against IFN-stimulated WT Balb/c rTECs was measured on d 5 of coculture. (F) CBA splenocytes were stimulated with irradiated B6 or CD112 KO splenocytes. Cytotoxicity against IFN-stimulated WT B6 rTECs was measured on d 5 of coculture.

Allospecific T cell cytotoxicity against rTECs is independent of DNAM-1 *in vitro*

RTECs act as non-professional APCs under inflammatory conditions (Starke *et al.* 2007; Wuthrich *et al.* 1990). We therefore wanted to test the importance of DNAM-1 signaling in allospecific cytotoxic activity against rTECs during the effector phase. Therefore, we used allospecifically stimulated splenocytes and tested their cytotoxic activity against WT and CD155 or CD112 KO rTECs. In both cases, when taking away one ligand on the target cells, killing rates were not altered (Fig. 2.15A and B). As there could be redundancy between both ligands, we used CD155 KO targets and added a blocking antibody against CD112 during the effector phase. Also, when none of the two ligands was available for DNAM-1, rTEC killing was not impaired (Fig. 2.15C). Finally, to exclude the possibility of a third unknown ligand binding to DNAM-1 we added a blocking antibody against DNAM-1. This also did not reduce allospecific killing of rTECs (Fig. 2.15D), suggesting that DNAM-1 activity is not essential for the cytotoxic activity of T cells against rTECs *in vitro*.

Lack of CD155 or CD112 expression does not prolong allogeneic skin graft survival

In order to evaluate the role of CD155 and CD112 for allograft rejection *in vivo*, we performed skin grafts using grafts from donors deficient in either CD155 or CD112 on completely MHC mismatched recipients. In both cases, no difference in the time course of graft rejection could be observed (Fig. 2.17A and C). To look for more subtle differences we repeated these experiments in a minor-mismatch and a single-antigen-mismatch situation. For the CD155 KO donors, we used DBA/2 mice. These mice possess the same haplotype as Balb/c (H-2^d), but have a different background leading to a mismatch in minor antigens. CD112 KO animals are of B6 background (H-2^b). In this case we used bm1 mice as recipients. These animals have a three amino acid exchange in the K^b molecule, leading to a single-antigen-mismatch compared to WT B6. In both combinations, no prolongation of skin graft survival was detected, when one of the two ligands for DNAM-1 was absent on the graft (Fig. 2.16C and D). In contrast, when CD112 was missing on the allograft, survival was even impaired (Fig. 2.16D).

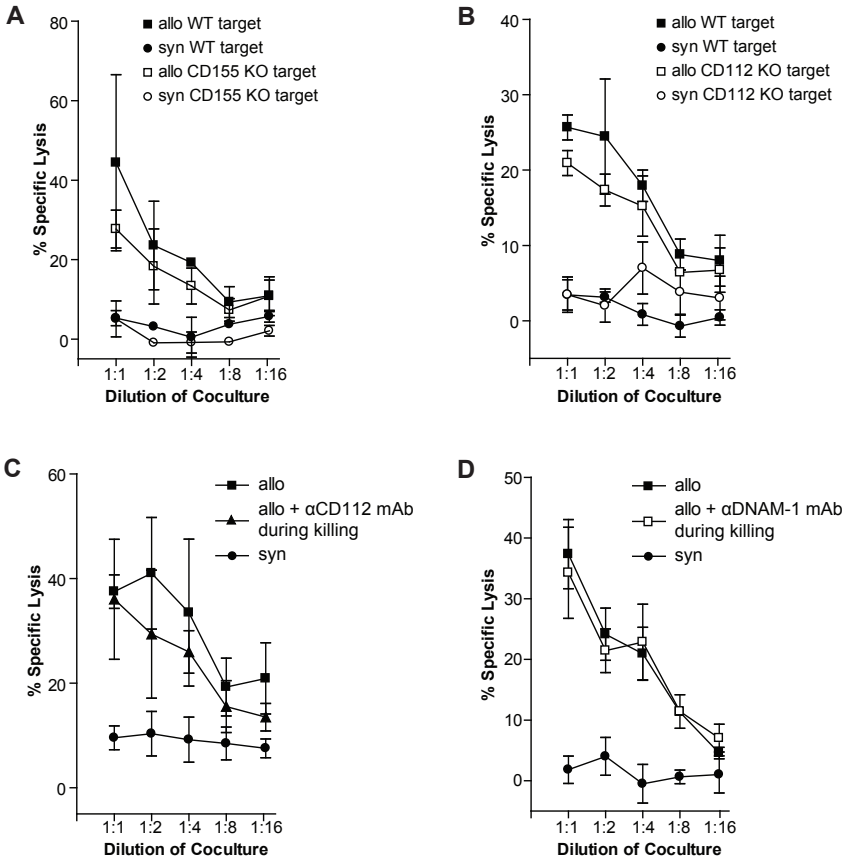

Figure 2.15: Cytotoxic activity of allospecific T cells against rTECs is not DNAM-1 dependent *in vitro*. (A) CBA splenocytes were stimulated with irradiated Balb/c splenocytes. Cytotoxicity against IFN-stimulated WT Balb/c or CD155 KO rTEC targets was measured on d 5 of coculture. (B) CBA splenocytes were stimulated with irradiated B6 splenocytes. Cytotoxicity against IFN-stimulated B6 WT or CD112 KO targets was measured on d 5 of coculture. (C) CBA splenocytes were stimulated with irradiated Balb/c splenocytes. Cytotoxicity against IFN-stimulated CD155 KO rTEC targets in the presence (25 μg/ml) or absence of a blocking anti-CD112 antibody was measured on d 5 of coculture. (D) CBA splenocytes were stimulated with irradiated B6 splenocytes. Cytotoxicity against IFN-stimulated WT targets was measured on d 5 of coculture in the presence or absence of a blocking anti-DNAM-1 antibody (50 μg/ml).

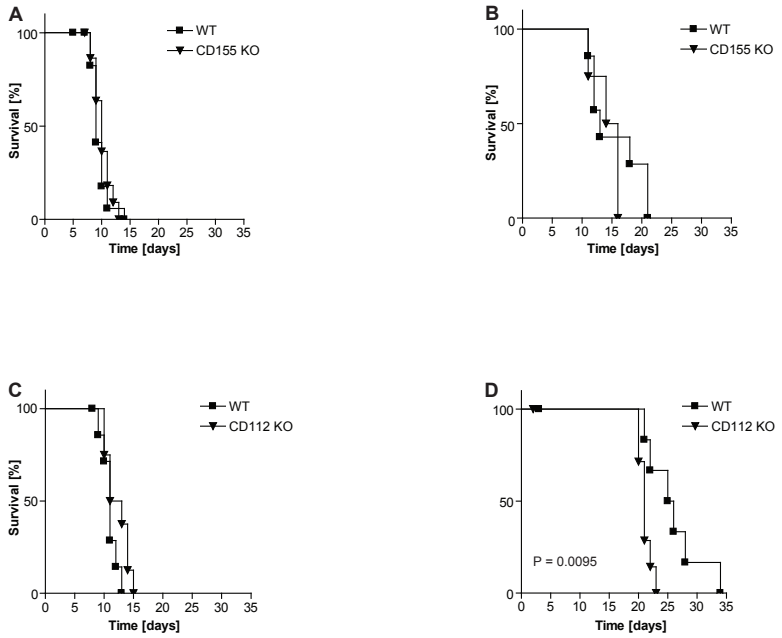

Figure 2.16: Lack of DNAM-1 ligands does not prolong skin allograft survival. (A) Skin grafts from fully MHC-mismatched WT Balb/c ($n = 19$) or CD155 KO ($n = 24$) donors were performed on B6 recipients. Median survival time: 9 vs. 10 days (WT vs. CD155 KO). (B) Skin grafts from WT Balb/c ($n = 7$) or CD155 KO ($n = 8$) donors were performed on DBA/2 recipients. Median survival time: 13 vs. 15 days (WT vs. CD155 KO). (C) Skin grafts from fully MHC mismatched WT B6 ($n = 8$) or CD112 KO ($n = 8$) donors were performed on CBA recipients. Median survival time: 11 vs. 12 days (WT vs. CD112 KO). (D) Skin grafts from single antigen mismatched B6 ($n = 7$) or CD112 ($n = 8$) donors were performed on bm1 recipients. Median survival time: 25.5 vs. 21 days (WT vs. CD112 KO), $P = 0.0095$ in log-rank-test.

Renal allograft rejection is not dependent on DNAM-1 ligand expression in the graft

Finally, we explored if expression of either ligand for DNAM-1 has an influence on renal allograft rejection *in vivo*. We thus performed kidney allografts in a completely MHC- mismatched model. For CD155 KO we used the combination Balb/c to B6 and for CD112 B6 to CBA was used. Renal allografts were performed in a non-life supporting manner, leaving the left kidney in place and replacing only the right kidney by the allograft. After 21 days allografts were harvested. All allografts showed severe infiltrates in the interstitium independent of expression of CD155 or CD112. After 21 days the renal parenchyma showed intense interstitial infiltrates, important tubulitis, endothlitis, and tissiue destruction in all allografts (Fig. 2.17A-B, D-E).
Interstingly, we observed a higher incidence of infarcts and subsequent development necrosis in allografts lacking CD155 or CD112. The exact reason for this finding is still to be elucidated.
We also assessed the amount of apoptotic epithelial cells in all allografts, as an indirect measure of cytotoxic activity *in vivo*. Also for this parameter we could not detect any difference when comparing CD155 or CD112 KO allografts with the matching WT controls, confirming our *in vitro* results (Fig. 2.17C and F).

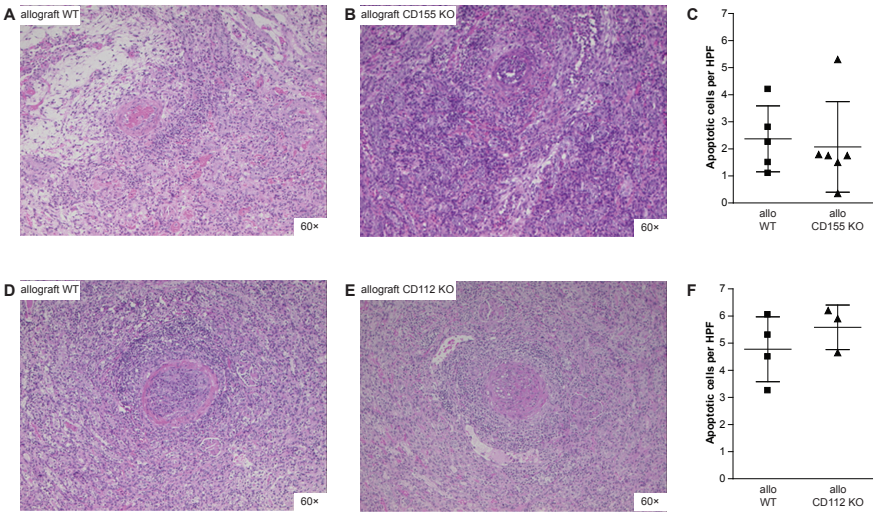

Figure 2.17: Kidney grafts. (A-C) Balb/c WT or CD155 KO mice were used as donors for non-life-supporting kidney grafts to B6 recipients. Organs were harvested after 21days ($n = 5$ per group). (A, B) Representative hematoxylin and eosin stain showing interstitial infiltrates and parenchymal destruction in WT (A) and CD155 KO (B) allografts. (D-F) B6 WT ($n = 4$) or CD112 KO ($n = 3$) mice were used as donors for non-life-supporting kidney grafts to CBA recipients. Organs were harvested after 21days. (D, E) Representative hematoxylin and eosin stain showing interstitial infiltrates and parenchymal destruction in WT (D) and CD112 KO (E) allografts. (C, F) Immunohistochemistry for ssDNA was performed on all grafts. Quantitative analysis is depicted.

Discussion

In this study we evaluated the role of DNAM-1 and its ligand for allospecific T cell activation in *in vitro* and *in vivo*. We found that DNAM-1 signaling played a role during the priming of directly alloreactive T cells *in vitro*. However, this process was independent of the two known ligands of DNAM-1, CD155 and CD112. Furthermore, we could not detect a role for DNAM-1 signaling during the effector function of cytolysis of rTECs mediated by allospecific T cells. Using two *in vivo* models for allograft rejection, skin and renal allografts, these *in vitro* results were confirmed. The time course of skin allograft rejection was not altered, depending on the expression of CD155 or CD112. Also the severity of cellular rejection in renal allografts from CD155 or CD112 KO donors was similar to that in WT grafts. However, in renal allografts from CD155 or CD112 KO donors a higher incidence of infarcts was detected.
Expression of CD155 and CD112 in murine kidneys has previously been shown (Nabekura *et al.* 2010). Both molecules are expressed on epithelial and endothelial cells (Takai *et al.* 2008). Here we show that CD155 and CD112 were upregulated in acutely rejected renal allografts. The exact site of expression cannot be identified by qPCR. Immunohistochemical staining is currently performed. However, we found expression of CD155 and CD112 on primary rTECs, which was increased under inflammatory conditions, indicating expression of the two molecules to the tubular compartment of the kidney.
In several *in vitro* models it was shown, that the expression of CD155 or CD112 facilitates the DNAM-1 mediated cytotoxic activity of NK and T cells against tumor cells (Shibuya *et al.* 1996; Tahara-Hanaoka *et al.* 2006) or autologous DCs (Pende *et al.* 2006; Seth *et al.* 2009). This is also represented by the lower ability of DNAM-1 deficient mice to reject tumors (Iguchi-Manaka *et al.* 2008). Furthermore, Gilfillan *et al.* postulated a role of DNAM-1/CD155 interaction for the cytotoxic activity of T cells against non-professional APCs (Gilfillan *et al.* 2008). We thus hypothesized that the interaction of T cells with rTECs, which are capable of acting as non-professional APCs and do express both CD155 and CD112, might also be dependent on DNAM-1 interactions with its ligands. However, we could not detect any role for DNAM-1 ligation by CD155 or CD112 for cytotoxic activity against allogeneic rTECs *in vitro* and *in vivo*.
In the study of Gilfillan *et al.* it was also shown that OVA-specific DNAM-1 expressing CD8 T cells proliferated more when stimulated with an OVA-pulsed, CD155 transformed T cell lymphoma cell line (EL-4), compared to their DNAM-1 KO counterparts (Gilfillan *et al.* 2008). Consistent with this we saw reduced proliferation and subsequent lower cytotoxic activity against rTECs, when DNAM-1 was blocked during the stimulation of T cells. However, this observation was completely independent of the expression of DNAM-1 ligands on the stimulator or target cells. Furthermore, DNAM-1 was dispensable for cytotoxic activity against rTECs of fully activated allospecific T cells. This suggests a role for DNAM-1 signaling during T cell priming, and not during the process of cytolysis in this *in vitro* model of alloreactivity. Moreover, this signaling was independent of interaction of DNAM-1 with CD155 or CD112, indicating another role for DNAM-1 in T cell priming. In line with this Shibuya

et al. showed that DNAM-1 signaling is involved in the LFA-1 mediated T cell activation (Shibuya *et al.* 2003). The authors induced T cell proliferation and differentiation by stimulating CD3 and LFA-1 and showed that this process is dependent on DNAM-1 signaling. In these experiments functional DNAM-1 was crucial for T cell activation - completely independent of ligand interaction and only dependent on the interaction with LFA-1 and phosphorylation of tyrosine 322 in DNAM-1 (Shibuya *et al.* 2003). Direct interaction of DNAM-1 and LFA-1 was proven by co-precipitation experiments (Shibuya *et al.* 2003).
In a previous report it was postulated that a role for DNAM-1 signaling for the activation of T cells by professional APCs like DCs can be excluded (Gilfillan *et al.* 2008). However, in our *in vitro* model T cells are mainly activated by DCs (Kraus *et al.*, submitted manuscript). The first interaction of T cells with DCs is mediated by LFA-1 binding to ICAM-1 (Ford and Larsen 2009). The blockade of DNAM-1 with an antibody might prevent close interaction of DNAM-1 and LFA-1 and thus prevent the costimulatory signal, which is mediated by LFA-1. Moreover, the bound antibody might lead to a steric inhibition of the T cell-APC contact at the site of LFA-1/ICAM-1 interaction and thereby impair efficient T cell priming.
When using CD155 or CD112 deficient cells as stimulators in *in vitro* proliferation and cytotoxicity assays, we could not detect reduced T cell activation. In contrast, T cell effector functions were, if at all different to the WT control, rather increased. This effect was the same for CD4 and CD8 T cells and also, when they were cocultured. This observation indicated a regulatory role of CD155 and CD112 during the process of T cell priming. Indeed, it has been shown that CD155 on human vascular endothelial cells attenuates the acquisition of effector functions in CD8 T cells (Escalante *et al.* 2011). Furthermore, another receptor for CD155 was recently identified to be expressed on CD4 T cells: TIGIT binds to CD155 and thereby reduces naive human CD4 T cell activation (Yu *et al.* 2009). Finally, CD155 KO mice have been shown to be more susceptible to graft-versus-host disease in a CD4 T cell-dependent model (Seth *et al.* 2011). The exact mechanisms for this phenomenon have still to be elucidated.
A recent study demonstrated that DNAM-1 blockade or deficiency on T cells reduced graft-versus-host disease in a CD8 T cell-dependent model (Nabekura *et al.* 2010). We thus wanted to test the role of the two DNAM-1 ligands in solid allograft rejection. Therefore we used two different models. Skin grafting is the most stringent model for allograft rejection. In this setup, we could not show an influence for any of the two known DNAM-1 ligands. This was true for both, major- and minor-mismatch models. For CD155 we also showed that it is dispensable for both the priming and the effector phase (data not shown). We further tested our findings in a completely MHC mismatched renal allograft model. DNAM-1-CD155 interaction is crucial for the diapedesis step during monocyte extravasation in a model using a human endothelial cell line (Reymond *et al.* 2004). One would therefore suspect to find a lower amount of infiltrating cells in renal allografts from CD155 KO donors. We could not detect such an effect. WT and CD155 KO allografts showed similar amounts of cellular infiltrates in the interstitium, and the same was true for CD112 KO

allografts. This finding goes along with the report of Seth *et al.*, who could not detect differences in infiltrates in the intestine after induction of graft-versus-host-disease in CD155 KO animals (Seth *et al.* 2011). We found that the same is also true for CD112 KO kidney allografts.
DNAM-1 mediates platelet adhesion to vascular endothelial cells (Kojima *et al.* 2003). One could therefore speculate that binding of platelets to endothelium lacking CD155 or CD112 might be reduced. However, one difference that we found between renal allografts from WT donors and those deficient in CD155 or CD112 was a higher incidence of infarcts, when one of the two molecules was missing. Taking into account the pivotal role, which nectin and necl proteins play for cell-cell adhesion, a less intact endothelium might be the reason for more thrombosis. CD155 is located on the leading edge of moving cells and mediates signaling to inhibit proliferation upon cell-cell-contact (Takai *et al.* 2008). CD112 together with other nectin proteins builds first cell-cell adhesion connections before cadherins (Takai *et al.* 2008). Ischemia-reperfusion injury causes endothelial damage (Harris *et al.* 1996), which might be repaired less efficiently in renal allografts from CD155 and CD112 KO donors. Gaps in the endothelium might then reveal the subendothelial layer and allow platelet activation and adhesion leading to clotting of the vessel (Wagner and Burger 2003). This hypothesis needs further investigation.
Taken together our results suggest a beneficial effect in solid allograft rejection for DNAM-1 blockade by reducing T cell priming. The blockade of either one of its ligands, however, does not seem favorable due to increased T cell activity *in vitro*, no effect on renal allograft rejection, and higher incidence of thrombosis.

Chapter 3

Discussion

The role of CD40-CD154 during allospecific CD8 T cell priming

The classical model for CD4-dependent CD8 T cell activation includes the interaction of both the CD4 and the CD8 T cell with the same APC. The CD4 T cell interacts with the DC via CD154-CD40 and thereby enables cross-presenting of antigens and higher expression of costimulatory molecules like B7 (Banchereau and Steinman 1998). Such a maturated DC is then able to efficiently prime CD8 T cells (Fig. 3.1). The CD4 T cell on the other hand is enabled to produce cytokines like IL-2, which gives a survival signal to the CD8 T cell. That this model is true also for alloresponses is supported by the finding, that triggering of CD40 can replace CD4 help in a murine model for heart transplantation (Fischbein *et al.* 2000), meaning that the sole need for CD8 activation is a DC matured due to CD40 ligation. Shepherd *et al.* however used a model, in which the allospecific clearance of injected P815 cells was not restored by exogenous CD40 triggering in CD154 deficient mice. They furthermore could show that APCs in CD154-deficient mice did not show signs of reduced activation. This suggests, that the lack of CD154 on T cells disables acquisition of T cell effector function, even though DCs would be well able to induce it. In our *in vitro* studies we also could not detect a difference between WT and CD40 KO DCs in MHC class II and B7 molecule expression after coculture with allogeneic T cells (data not shown). Thus, the defect in cytotoxic T cell priming, which we observed *in vitro* when APCs did not express CD40, is not due to reduced DC maturation, but to a lack in CD154 signaling to the T cell itself.
A direct interaction of CD154 on CD8 T cells and CD40 on the DC is not included in the classical model of CD40-mediated DC maturation and T cell priming (Fig. 3.1). However, CD154 is expressed on CD8 T cells (Hermann *et al.* 1995; Sad *et al.* 1997). When using a skin graft model, which is mainly restricted to the response of directly alloreactive CD8 T cells due to a single MHC class I mismatch, we detected prolongation of skin graft survival, if the donor did not express CD40. This indicates that also CD4- independent CD8 T cells need a costimulatory signal via CD154. It is known that CD154-deficient mice mount reduced allospecific immune responses (Buhlmann and Noelle 1996; Zhai *et al.* 2002). However, in these models a deficient CD4 T cell help cannot be ruled out as reason. Other group's results support our findings by using CD4-depleted recipients for completely MHC-mismatched skin allografts. Blocking CD154 with an antibody in this model prevented CD4 T cell-independent CD8 activation (Zhai *et al.* 2003). It has furthermore been shown that CD4 T help-independent clearance of allogeneic cells happened, when injected P815 cells were expressing B7-2. Injecting such cells in CD154 deficient animals however did not lead to generation of allospecific CD8 positive CTLs. This could also not be restored by maturating host DCs by exogenous triggering of the CD40 pathway (Shepherd and Kerkvliet 1999). This suggests, along with our results, that a direct interaction of CD8-CD154 and APC-CD40 is necessary for alloreactivity. In contrast, there are reports claiming, that allospecific CD8 T cells are not sensitive to CD154 blockade and still are able to reject skin allografts

independently of CD40-CD154 signaling (Jones *et al.* 2000). However, they were at least partially performed with T cell receptor transgenic CD8 T cells from BM3.3 mice, which are easy to activate and do not depend on CD8 binding. We have used these cells in *in vitro* experiments and found that they are, in contrast to WT T cells, not CD40-dependent (data not shown).
Moreover, we could show *in vitro* for the first time that directly allospecific CD8 T cells need a direct signal via CD154 to achieve full activation, even in the presence of intact CD4 help (Fig. 3.1). This result has still to be proven *in vivo*.

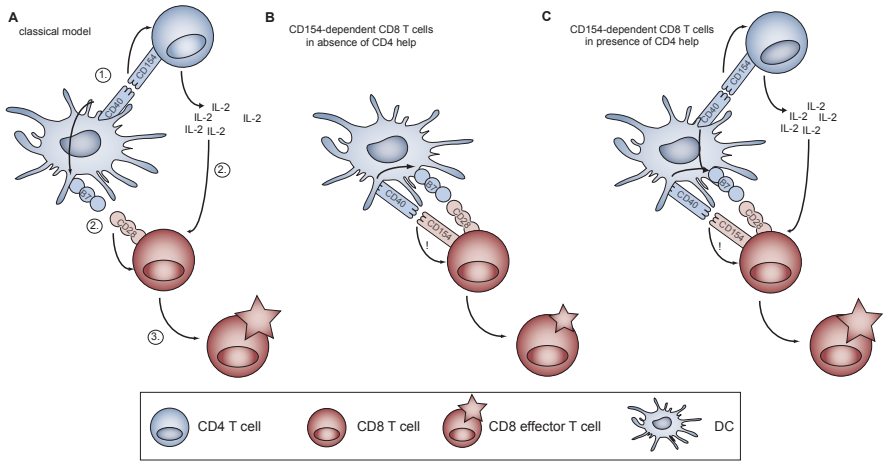

Figure 3.1: Models for CD8 T cell priming
(A) The classical model for CD4 dependent CD8 T cell activation includes the contact of CD4 and CD8 T cell with the same DC: (1.) Contact via CD154 on the CD4 T cell and CD40 on the APC enables the DC to express additional B7 molecules and to cross-present (2.). The CD4 T cell is enabled to produce IL-2, which gives a survival signal to the CD8 T cell (2.). Thus, a fully activated effector CD8 T cell is generated (3.). (B) In the absence of CD4 help, allospecific CD8 T cells need additionally to the B7-CD28 interaction a direct signal via CD154 in order to become potent effector cells. (C) Our *in vitro* results also support this third model, in which CD8 T cells are dependent on a direct interaction with the APC via CD154 in order to become completely activated, when CD4 help is intact.

Is Blockade of CD40 instead of CD154 as efficient?

Blockade of the CD40-CD154 pathway has so far mainly been performed by blocking CD154 with antibodies. The mechanism of immunosuppression observed with such agents is still not completely elucidated. While blocking CD154 might just be a way to block a costimulatory signal to the T cell, it has also been proposed that it leads to T cell apoptosis (Blair *et al.* 2000) or depletion (Monk *et al.* 2003). Therefore the ability of CD40-blockade to reproduce the same effects of CD154-blockade is questioned. There are some studies targeting CD40 for immunosuppression or tolerance induction in transplantation models in non-human primates. However, most of the antibodies used so far have shown at least partially B cell depleting properties (Adams *et al.* 2005;

Aoyagi *et al.* 2009; Pearson *et al.* 2002; t Hart *et al.* 2005). One of these antibodies (4D11, ASKP1240) is now in Phase II studies for kidney transplantation (Pilat *et al.* 2011). Just recently, two studies using a non-depleting anti-CD40 antibody were published. 3A8 is a completely murine antibody, which is able to block costimulatory signals to allospecific T cells, however still allows binding of soluble CD154 and induces B7 upregulation on B cells (Badell *et al.* 2012). It was able to prolong islet allograft survival and assist in induction of mixed chimerism combined with CTLA4-Ig and an mTOR inhibitor (Badell *et al.* 2012; Page *et al.* 2012). In recipients of islet transplants 3A8 was furthermore able to prevent development of allospecific antibodies. Even though these results are promising the potential immunogenicity of a fully murine antibody seems to hamper its *in vivo* efficacy (Badell *et al.* 2012).
Currently, there is no antibody against murine CD40 with similar properties. For mechanistic studies to elucidate the role of CD40 blockade compared to CD154-blockade such a tool would be very useful. In this study we therefore developed an F(ab)-fragment directed against murine CD40, which has shown potent inhibitory properties *in vitro* by inhibiting CD40-triggered B cell activation and proliferation and also allospecific T cell proliferation and cytokine production. The direct comparison between F(ab)86 and CD154-blockade still has to be made *in vitro* and *in vivo*. F(ab)86 has to be modified to increase half-life in order to be used *in vivo*. The strategies to do so are discussed in section 2 of the results chapter. Using an F(ab)-fragment, which is pegylated or fused to a larger protein like α1-antitrypsin, we also expect lower immunogenicity and a lower risk of adverse events usually linked to use of therapeutic antibodies in humans.

Which role do Th17 cells play in renal allograft rejection?

In vitro we found a strong dependency of allospecific Th17 induction on the costimulatory molecule CD40 expressed on the stimulator cells. This finding is supported by observations made by Iezzi *et al.* (Iezzi *et al.* 2009). They showed complete abolishment of Th17 induction in EAE using CD40-deficient mice. Thus, the use of costimulation blockade directed against the CD40 pathway might reduce the induction of the Th17 T cell subset, which has been shown to have deleterious effects in autoimmune diseases (Hu *et al.* 2010). However, the role of Th17 cells in allotransplantation is still under investigation. There are studies indicating a role for IL-17A in CD4-mediated graft-versus-host disease (Kappel *et al.* 2009) and in early allograft inflammation in a cardiac allograft model (Gorbacheva *et al.* 2010). Furthermore, increased IL-17 mRNA levels have been found early in human and rat acute renal allograft rejection (Loong *et al.* 2002).
The most convincing studies showing that IL-17-producing T cells can cause allograft rejection were performed in mice lacking the Th1-specific transcription factor T-bet (Burrell *et al.* 2008; Yuan *et al.* 2008). T-bet knock-out mice are resistant to costimulation blockade-induced tolerance. In one model this has

been shown to be due to IL-17 producing CD8 T cells (T17), which are polarized under similar conditions as Th17 cells. Blockade of IL-17 concomitant with costimulation blockade was able to induce long term survival of cardiac allografts (Burrell *et al.* 2008). Also blockade of TIM-1, which is expressed on T17 cells, could abrogate the otherwise costimulation-resistant alloresponse (Yuan *et al.* 2009). However, it remains unclear whether the processes observed in these studies are also taking place in animals sufficient in Th1 responses - especially because CD154-blockade induced allograft tolerance has been shown to be dependent on IFN-γ in a mouse model for cardiac transplantation. IFN-γ knock-out mice showed lower Treg activity in the allograft represented by lower intragraft FoxP3 and IL-10 expression (Jiang *et al.* 2010).
When searching for IL-17A expression in acutely rejecting renal allografts, we detected only very low amounts. This may be explained by various reasons:
First Iezzi *et al.* have shown that the induction of Th17 responses is dependent on antigen dose and toll-like receptor (TLR) triggering (Iezzi *et al.* 2009). In our kidney allograft model the amount of donor APCs migrating to the draining lymph node may be rather low in comparison to the amount of APCs in an *in vitro* MLR. Furthermore, the APCs in the *in vitro* MLR are irradiated and thus might receive a danger signal similar to triggering a TLR. Indeed, TLR-triggering simultaneously with cardiac transplantation is able to abrogate CD154-blockade induced allograft tolerance and induce rejection. This process has been shown to be dependent on IL-6 and IL-17 (Chen *et al.* 2009). In allografts undergoing rejection IFN-γ- and IL-17-producing CD4 T cells were found (Chen *et al.* 2009). We however did not add a TLR stimulus in our renal allograft model.
Second, it has been found in an *in vitro* study that human rTECs do not secrete MIP-3 under inflammatory conditions, a chemokine crucial for the recruitment of Th17 cells (Demmers *et al.* 2011). Thus, a low recruitment of Th17 cells to murine renal allografts could explain the rather low mRNA expression of IL-17A.
Third, Loong *et al.* detected IL-17 producing mononuclear cells in renal allografts as early as day 2 after transplantation (Loong *et al.* 2002). Since we harvested the renal allografts only at day 7, this time point might be too late to detect these effects. In fact, it has been shown that early Th17 migration into the lung is needed for subsequent Th1 accumulation in a murine model for tuberculosis (Khader *et al.* 2007). Furthermore, stimulation with IL-17A has been shown to induce chemokine production by rTECs (Woltman *et al.* 2000), which may trigger Th1 recruitment to the transplanted organ (Fig. 3.2) (Turner *et al.* 2001). Interestingly, it has been shown that the Th17 lineage is not completely committed. IFN-γ IL-17A double producers have been detected in the inflamed CNS (Damsker *et al.* 2010). Furthermore, Th17 cells can be converted to Th1 cells *in vitro* and *in vivo* (Damsker *et al.* 2010; Hirota *et al.* 2011). Th1 cells on the other hand do not seem to be that promiscuous and seem rather stable in their phenotype (Damsker *et al.* 2010). Considering this, it might also be possible that the Th17 cells needed for subsequent Th1 recruitment to the organ, already resemble part of these Th1 cells as they convert after time (Fig. 3.2).

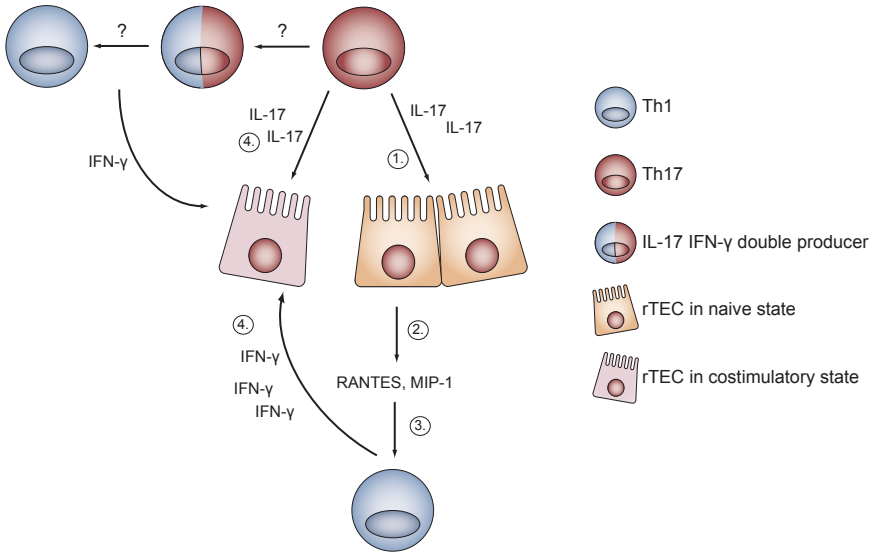

Figure 3.2: Early Th17 invasion into the renal allograft might be needed to enable subsequent Th1 infiltration. In this model Th17 cells are the first Th cells to encounter the allograft (1.). IL-17A production stimulates rTECs to produce chemokines (2.) that in turn facitlitate the recruitment of Th1 cells (3.) IFN-γ produced by Th1 cell together with IL-17 from Th17 cells leads to conversion of rTECs to a rather costimulatory surface expression pattern by downregulation of PD-L1 and upregulation of CD40 (4.). It might be possible that Th17 cells might convert to Th1 T cells over time via a state of IL-17 IFN-γ double production and thereby add to or resemble the pool of Th1 cells later present in the graft.

We also examined the response of rTEC surface expression induced by IL-17A stimulation. When stimulating rTECs with IL-17A alone, we did not detect any difference in surface marker expression (data not shown). However, an effect that may play a relevant role during renal allograft rejection was seen, when IL-17A was combined with IFN-γ. The two cytokines in combination induced a significant downregulation of the coinhibitory ligand PD-L1, which has been shown to protect human rTECs from allospecific CTL activity (Starke *et al.* 2010). On the other hand the expression of the costimulatory molecule CD40 was induced under the influence of both cytokines. This surface expression pattern makes rTECs even more attractive targets for allospecific T cell activity. Thus, the early involvement of IL-17 in renal allografts might play a role for rTEC modulation in the presence of a concomitant Th1 response, which does not take place in T-bet deficient animals.

Other costimulation pathways play a role in solid organ allograft rejection

Apart from the CD40-CD154 pathway the other costimulatory pathways play a variable role in induction of allograft rejection or allograft tolerance. The best characterized costimulatory pathway is the one mediated by CD28. Its ligation by the B7 molecules triggers IL-2 production and CD25 expression. The subsequent binding of IL-2 to CD25 triggers the mammalian target of rapamycin (mTOR), the target of some immunosuppressive drugs (Halloran 2004). The B7-molecules, however, also bind to the coinhibitory receptor CTLA-4 with a much higher affinity than CD28 (Linsley *et al.* 1994). A fusion protein of CTLA-4 and the Fc region of IgG1 (CTLA-4-Ig) was developed in order to block CD28 signaling (Linsley *et al.* 1992). CTLA-4-Ig has been used in several rodent models for solid organ transplantation. It induces long-term allograft acceptance of heart (Baliga *et al.* 1994; Turka *et al.* 1992), islet (Lenschow *et al.* 1992), and renal allografts (Azuma *et al.* 1996). Despite these strong effects, CTLA-4-Ig is not able to prevent completely MHC-mismatched skin graft rejection (Larsen *et al.* 1996). Also the translation of CTLA-4-Ig into non-human primates only leads to modest prolongation of survival of renal and islet allografts (Kirk *et al.* 1997; Levisetti *et al.* 1997). The CTLA-4-Ig molecule has been further developed to increase affinity to B7-1 and B7-2 and is now used in clinics as belatacept for maintenance treatment in renal transplant patients (Vincenti *et al.* 2010; Vincenti *et al.* 2005).
CTLA-4 is a coinhibitory receptor expressed on activated T cells and downregulates T cell responses (Walunas *et al.* 1994). Furthermore, CTLA-4 is constitutively expressed on Tregs and is critical for their suppressor function (Wing *et al.* 2008). Administration of CTLA-4-Ig prevents binding of B7 molecules to CTLA-4 expressing T cells. Moreover, it has recently been shown, that PD-L1 also binds to B7-1, and thereby suppresses T cell proliferation (Butte *et al.* 2007). Masking B7-1 with CTLA-4-Ig prevents also this interaction. Therefore, efforts are still made for developing a CD28-specific blockade, despite catastrophic results obtained for an anti-CD28 antibody in a phase I trial (Suntharalingam *et al.* 2006). Patients suffered from a massive cytokine storm upon administration of this antibody. To prevent this, monovalent molecules against CD28 are now developed. A single chain Fv construct with α1-antitrypsin directed against CD28 has been shown to prevent allospecific T cell activation *in vitro* (Vanhove *et al.* 2003) and to prevent renal and heart allograft rejection in combination with a calcineurin inhibitor in non-human primates (Poirier *et al.* 2010). Together with blockade of the CD40 pathway however, CTLA-4-Ig is able to induce long term allograft acceptance in a heart allograft model in mouse (Larsen *et al.* 1996), emphasizing the importance of these two costimulation pathways for allospecific immune responses.
However, in CD28-CD154 double knock-out mice complete MHC mismatched skin allografts are still rejected (Demirci *et al.* 2004). This rejection has been shown to be dependent on the OX40 costimulatory pathway. OX40 is expressed on activated T cells, preferentially on CD4 T cells (Gramaglia *et al.* 1998). Its

ligand OX40L is expressed on activated DC, B cells and vascular endothelial cells (Stuber and Strober 1996). The OX40/OX40L interaction is very important for the induction of T cell-dependent humoral responses (Stuber and Strober 1996). OX40 blockade alone is not very efficient in prolonging allograft survival (Pilat *et al.* 2011) and has little effect on primary T cell responses (Demirci *et al.* 2004). However, OX40-ligation is important for the generation of memory T cells and the survival of activated memory T cells (Dawicki *et al.* 2004).

Another costimulatory pathway, whose blockade is beneficial in solid organ transplantation, is the ICOS/ICOS-L pathway. ICOS is expressed upon activation on CD8 and CD4 T cells and persists on effector and memory T cells. ICOS blockade alone does prolong cardiac allograft survival to a lesser degree than antibodies against CD154 or CTLA4-Ig. However, when combined with either of them it leads to long term acceptance of cardiac allografts and prevents acute and chronic rejection (Ozkaynak *et al.* 2001). This is interesting, because chronic rejection still takes place in CD154 deficient animals (Shimizu *et al.* 2000). Furthermore, it has been shown that ICOS stimulation does maintain late CD154 expression on T cells (Kaminski *et al.* 2009).

The 4-1BB pathway is also associated with the CD40 pathway. 4-1BB is expressed on activated T cells, NK cells and DCs and its ligand 4-1BBL on DCs, macrophages, and B cells (Wang *et al.* 2003). The engagement of 4-1BB has been shown to be more important for CD8 activity than for CD4 T cells (Wang *et al.* 2003). The link to CD40 signaling has been found in a model system for CD8-dependent tumor rejection. Mice challenged with tumor cells were able to reject those, when 4-1BB was activated with an agonistic antibody. However, this was completely abolished in CD40 deficient mice (Miller *et al.* 2002). Indeed, CD40 ligation leads to the expression of 4-1BBL on DCs (Futagawa *et al.* 2002). Thus, CD40 signaling may be needed to induce complete activation of CD8 T cells via the 4-1BB pathway. In a model for intestinal transplantation it was shown that blockade of 4-1BB was able to prevent CD8 T cell-mediated rejection (Wang *et al.* 2003). Interestingly, 4-1BB blockade did not prevent allospecific CD8 T cell proliferation but reduced IFN-γ and TNF-α production in recipient spleens, indicating that 4-1BB signaling is not necessary for CD8 priming, but rather for the acquisition of effector function.

Finally, cell adhesion molecules have been shown to act as costimulatory receptors. LFA-1 expressed on T cells is such a molecule. It is crucial for T cell trafficking but also for the formation of the immunologic synapse between the APC and the T cell (Nicolls and Gill 2006). Furthermore, it is able to transduce positive signals to the T cell. This has been shown to be dependent on DNAM-1 (Shibuya *et al.* 2003). DNAM-1 is another cell-adhesion molecule, which has been shown to deliver costimulatory signals in T and NK cells. Its importance for immune responses against tumors mediated by NK cells but also CTLs is studied in greater detail than its role in alloimmunity. It has however been shown, that blocking DNAM-1 in a graft-versus-host model is beneficial (Nabekura *et al.* 2010). Similar results have been found for LFA-1 blockade alone and even stronger in combination with CD28 blockade (Blazar *et al.* 1995). In murine models for solid organ transplantation LFA-1 blockade

synergized with other costimulation blockers to induce long-term allograft survival of islets and cardiac allografts and even of immunologically challenging skin allografts (Pilat *et al.* 2011). The mechanisms how LFA-1 blockade prolongs allograft survival are not completely clear yet. It is likely though, that the effect is mediated by blockade of the costimulatory signal delivered by LFA-1 and not so much by blockade of T cell trafficking (Ford and Larsen 2009). It needs to be further investigated, to which degree LFA-1 and DNAM-1 signaling pathways are interdependent. It has been suggested that DNAM-1 signaling induced by ligand binding requires additional LFA-1 signals in T cells, which are not completely activated yet (Tahara-Hanaoka *et al.* 2004).
Furthermore, it is not completely clear which role the ligands of DNAM-1 play in alloresponses. The two molecules are constitutively expressed on many cell types and important for cell-cell adhesion (Takai *et al.* 2008). We found that DNAM-1 blockade reduces allospecific T cell priming independent of the two ligands for DNAM-1. In contrast, we found rather higher induction of alloresponses, when the stimulating cells were lacking CD155 or CD112 - a finding consistent with enhanced graft-versus-host diesease in CD155 KO recipients (Seth *et al.* 2011). Moreover, when we transplanted kidneys from CD155 or CD112 donors, we observed a higher incidence of thromboembolic events, indicating a less stable vascular endothelium in these kidneys. Research conducted to reduce allospecific response to solid organ transplants will thus rather have to focus on DNAM-1 in combination with LFA-1 and most probably not on the ligands for DNAM-1.

Costimulatory molecules expressed on rTECs

With this work we could offer a more detailed insight in the interaction of host T cells with donor APCs and rTECs via two costimulatory molecules. Interestingly, we could not detect a major influence of CD40, CD155, or CD112 expressed on rTECs on the activity of allospecific T cells, as it has been shown for the coinhibitory molecule PD-L1 (Starke *et al.* 2010).
For the ligation of CD40 on rTECs it is described, that rTECs increase chemokine production (Woltman *et al.* 2000). It would thus be interesting to investigate the results of CD40 ligation on rTECs for the microenviroment of a renal allograft in more detail. We found better renal function in allografts which did not express CD40. So far we linked this to a difference in the cytokine millieu in the allograft and subsequent alterations of rTEC surface expression. However, there might still be an additional regulatory influence of CD40 signaling in the rTEC, which we were not able to detect with the methods we used. Considering the much higher expression of CD40 on human rTECs (Demmers *et al.* 2011) the influence of rTEC-CD40 on the interaction with T cells might also differ greatly compared to the murine model system. In this context, blocking CD40 might be beneficial for protection of the renal allograft beyond inhibiting T cell responses, and in this respect also be an advantage over CD154 blockade.

About the importance of CD155 and CD112, the two ligands for DNAM-1, expressed on rTECs not much is known apart from their cell-cell-adhesion properties. We could not detect an immune-regulatory function of these two molecules expressed on rTECs in this work. However, CD155 expression on human endothelial cells has been shown to have an influence on the activation of CD8 T cells by these cells (Escalante *et al.* 2011). rTECs can also act as non-professional APCs. However, we were not able to induce T cell proliferation by stimulation with allogeneic rTECs, which is most probably due to the high PD-L1 expression induced by inflammatory stimuli. Thus, we were unfortunately not able to reproduce these results. During priming by professional APCs, however, CD40 and DNAM-1 play important roles. CD40 expressed on donor APCs is crucial for complete activation of directly alloreactive T cells including Th17, Th1 and CTLs.
Another costimulatory molecule expressed on rTECs is ICOS-L (Starke *et al.* 2007). Not much is known about its role for modulation of intrarenal immune responses. It has been suggested that ICOS-L on rTECs induces IL-10 secretion in infiltrating T cells (De Haij *et al.* 2005). Blockade of ICOS in a renal allograft model sustained the inflammatory response in the kidney and led to increased tissue damage (Lutz *et al.* 2007). These studies indicate that ICOS-L on rTECs rather mediates a downregulation of the immune responses in the kidney.
ICAM-1, the major ligand for LFA-1, is expressed on rTECs and upregulated under inflammatory conditions (Wuthrich *et al.* 1990). Lack of allograft ICAM-1 prolonges heart allograft survival. However, parallel to our results concerning DNAM-1, ICAM-1 expression was more imprtant on passenger APCs to induce T cell priming, not on the organ itself (Zhang *et al.* 2003).
Taking into account the variety of costimulatory and -inhibitory molecules expressed on rTECs and their different regulation, more research is needed in order to explore the way rTECs modulate the local intra-graft immune response during renal allograft rejection.

Memory T cells - a hurdle in solid organ transplantation

A state of donor specific tolerance in mouse models is nowadays easily achieved by induction of mixed hematopoetic chimerism via donor bone marrow transplantation under costimulatory blockade (mainly CD154 blockade) (Fehr *et al.* 2005; Wekerle *et al.* 1999). However, translation of these models into non-human primates led to prolonged allograft survival and reduction of alloimmunity, but failed to induce consistent tolerance (Ochiai *et al.* 2007). Memory T cells have been found to be a major obstacle for tolerance induction in non-human primates (Nadazdin *et al.* 2011). The composition of the T cell repertoire differs greatly between a laboratory mouse and a primate. Naive mice held under pathogen free conditions usually possess about 4 to 8% memory T cells whereas the T cell repertoire in an adult primate or human is composed of nearly 50% memory cells (Nadazdin *et al.* 2011). This memory T cell pool

also contains allospecific cells. The most obvious way to develop memory T cells specific for alloantigens is previous exposure to the antigen by pregnancy, blood transfusion, or previous transplantation (Bingaman and Farber 2004). In a murine model it has for example been shown that transfused platelets, which are not expressing MHC, can induce memory T cells against minor antigens that are able to reject subsequent bone marrow transplants (Patel *et al.* 2009). However, even persons who have never received a transplant before can show allospecific memory (Ford and Larsen 2011). The exposure to environmental pathogens has been shown to produce cross-reactive memory T cells, which are able to recognize foreign MHC molecules (Burrows *et al.* 1995; Pantenburg *et al.* 2002). For example T cells specific for Epstein-Barr virus nuclear antigen-3A in the context of HLA-B8 have been shown to also recognize HLA-B44 (McDonald *et al.* 2009). In fact, 45% of virus-specific T cell clones have been shown to be cross-reactive with at least one allogeneic MHC molecule (Amir *et al.* 2010). Interestingly, the frequency of alloreactive T cells is the same in all T cell compartments (naive, central memory, effector memory, and terminal effector memory) (Macedo *et al.* 2009). The difference between a primary and a memory response thus is not due to different frequencies but different qualities between naive and memory T cells (Ford and Larsen 2010). Memory T cells are relatively resistant to calcineurin inhibition and costimulatory blockade (Nadazdin *et al.* 2011) but also to depleting therapies (Pearl *et al.* 2005) and regulation (Yang *et al.* 2007). Thus, T memory cells are not only an obstacle for tolerance induction but also for long term allograft survival under immunosuppression. However, the ability of memory T cells to induce allograft rejection depends on the degree and duration of antigen exposure, context of antigen presentation, and type of pathogen infection (Ford and Larsen 2011). Furthermore, the affinity of the TCR expressed on the memory T cell to an allo-antigen might determine the possibility to tolerize the cell (Ford and Larsen 2011). It has been shown that the frequency of donor-specific memory T cells correlates with allograft outcome in a non-human primate model for renal transplantation in combination with induction of mixed chimerism (Nadazdin *et al.* 2011).

One option to avoid the barrier formed by donor-specific memory T cells would be a careful selection of recipient-donor pairs by choosing recipients with low memory for the respective donor. However, this is not too easy to implement considering the overall shortage of donor organs and the fact, that donor-recipient pairs are only chosen in advance in living donation. On the other hand, the evaluation of donor-reactive memory T cell frequency and the strength of their reaction might enable tailoring a more individualized immunosuppression according to the risk the recipient is at (Ford and Larsen 2011).

Another or an additional option is the use of new therapies directed specifically against memory T cells. Targeting CD2 or LFA-1 has been shown to prevent allograft rejection by memory T cells. CD2 is expressed on effector memory T cells, which are able to mediate costimulation blockade resistant allograft rejection. Blockade of CD2 in combination with CTLA-4-Ig and mTOR blockade resulted in renal allograft survival greater than 90 days even after discontinuation of treatment (Weaver *et al.* 2009). Memory T cells are able to traffic very

quickly into allografts (12 - 24 hours post transplantation) (Schenk *et al.* 2008). Blockade of LFA-1 with an antibody was able to prevent this early infiltration (Setoguchi *et al.* 2009) and was effective to inhibit donor-reactive memory T cell responses when combined with costimulation blockade (Kitchens *et al.* 2011). But one has to consider that long-term targeting memory T cell activity is on the expense of a loss of the memory response against environmental pathogens. However, in a non-human primate model for islet transplantation an approach to use LFA-1 induction therapy combined with belatacept seemed promising (Badell *et al.* 2010). Our findings about DNAM-1 for allospecific T cell priming and its interplay with LFA-1 (Shibuya *et al.* 2003) suggest that this molecule might also be a target for such a purpose.

Costimulation blockade in the clinics

Costimulation blockade for tolerance induction

The final goal of transplantation immunology research is to achieve a state of donor-specific tolerance, which is defined as lack of acute and chronic allograft rejection in the absence of ongoing immunosuppressive therapy and an intact immune reactivity to pathogens and cancer. With such a situation in a patient, it would be possible to avoid toxicities of current immunosuppressive therapies, opportunistic infections, and cancer. Thus, major obstacles for long term allograft survival that patients are facing today would be abolished. Costimulation blockade is a promising tool to be used together with DST or bone marrow transplantation in order to induce mixed chimerism in experimental models. However, it has been shown, that tolerance induction with DST under costimulation blockade is broken after time by new T cells leaving the thymus (Iwakoshi *et al.* 2001).

The mixed chimerism approach allows a more stable state of tolerance. The experimental protocols were translated into a clinical setting, leading to a study with five patients receiving combined bone marrow and kidney transplantation from HLA-mismatched donors (Kawai *et al.* 2008). Four of the five patients have accepted their renal allografts and are off immunosuppressive drugs since several years. The regimen these patients received contained cyclophosphamide for myelosuppression, anti-CD2 antibodies, cyclosporine A, and thymic irradiation for reduction and blockade of peripheral T cells prior to kidney transplantation and subsequent donor bone marrow transfusion (Kawai *et al.* 2008). After transplantation recipients received cyclosporine A for a period of some months. In some patients rituximab (a B cell depleting antibody), was added to the induction protocol and steroids were given for ten days following transplantation (Kawai *et al.* 2008). Interestingly, all patients lost chimerism over time, which did not inevitably lead to rejection of the kidney graft and reactivation of donor responsiveness. For the patients a life free of immunosuppressive drugs it is now a big advantage in quality of life and health.

On the other hand there are critics, who would not describe the state of these patients as one of a donor-specific tolerance. Halloran *et al.* suspect that the invasive induction therapy used here induces changes in the lymphoid system

that might last for years (Halloran *et al.* 2008). Thus, they speculate the patients still "suffer" from immunosuppression and therefore are not able to reject their kidney grafts and still remain at risk for infections or malignancies. If they are right, one will only be able to tell after time.
Indeed, it is true that the intense induction treatment has made the approach untranslatable to a larger patient cohort. Furthermore, the mixed chimerism approach in this setup is limited to living donation. Studies are now undertaken to solve this problem and transplant donor bone marrow at a later time point after transplantation (Koyama *et al.* 2007). Thus, new strategies to induce mixed chimerism in patients have to be evolved and costimulation blockade could be a part of them.

Costimulation blockade for maintenance immunosuppression

Costimulation blockade on the other hand is also an option for maintenance therapy in allograft recipients. With well designed biologics directed against costimulatory molecules some of the toxic effects of current therapy options could be avoided. An example for this is belatacept, which is used in clinics for renal transplantation in combination with mycophenolic acid and steroids in order to avoid calcineurin inhibitor toxicity. Phase III trials have shown reduced signs of calcineurin inhibitor nephrotoxicities after one year and improved kidney function compared to a control group receiving cyclosporine A even after 3 year follow-up (Vincenti *et al.* 2011). Also the incidence of new onset diabetes and dislipidemia were reduced in the groups not receiving cyclosporine A (Vincenti *et al.* 2011). Unfortunately in the belatacept treated groups a higher incidence of post-transplant lymphoproliferative disorder (PTLD) was observed. This was most elevated in the group receiving a high dose of belatacept and mainly restricted to Epstein-Barr virus negative patients receiving a graft from a seropositive donor, leading to B cell lymphomas (Vincenti *et al.* 2011).
Another risk factor is, as discussed above, that belatacept does not only block CD28 signaling but also CTLA-4 and PD-L1 signals, which are important for regulating T cell proliferation. Furthermore, CD28 signaling leading to IL-2 production is essential for the development of Tregs (Pilat *et al.* 2011). CTLA-4 is constitutively expressed on Tregs, and is critical for their suppressor function (Wing *et al.* 2008). Thus, belatacept in high doses might block Treg induction and activity. Finally CD28 signaling has been shown to prevent Th17 induction (Bouguermouh *et al.* 2009). Blocking CD28 might therefore lead to a stronger induction of this proinflammatory Th subset. Thus, the use of only CD28 blockade alone might not be the most efficient way to use costimulatory blockade in the future.
Combining belatacept with an agent blocking CD40 may prevent some of the mentioned adverse effects. Belatacept might be used in lower doses, when combined with CD40 blockade, thereby preventing complete masking of the beneficial effects of CTLA-4 on Tregs. Furthermore, as we and others have shown, Th17 induction is dependent on CD40 signaling (Iezzi *et al.* 2009), thus

might be reduced when blocking also the CD40 pathway. Additional to this, it might be useful to block a costimulatory pathway, which is important for T-B-cell interaction (e.g. CD40), to avoid the formation of *de novo* allospecific antibodies.
A blocking antibody directed against CD40 (4D11) is going to clinical phase II trials right now, and the results are going to give a deeper insight in the efficacy of this approach. Our development of a blocking F(ab)-fragment directed against murine CD40 might help alongside to understand the mechanistic differences between CD40 and CD154 blockade and develop new protocols for tolerance induction.

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